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Award Number: DAMD17-99-1-9024

TITLE: The Cell Cycle Inhibitor p27KIP1: A Key of G1 Arrest by Androgen Ablation and by Vitamin D3 Analog

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REPORT DATE: February 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2001	Final (04 Jan 99 - 03 Jan 01)

4. TITLE AND SUBTITLE
The Cell Cycle Inhibitor p27KIP1: A Key Mediator of G1 Arrest by Androgen Ablation and by Vitamin D3Analog

5. FUNDING NUMBERS
DAMD17-99-1-9024

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Please see next page

14. SUBJECT TERMS
Prostate cancer, cell cycle, androgen receptor, vitamin D3, hormone Resistance, dk inhibitor p27

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

13. ABSTRACT

Our previous research in LNCaP and *in vivo* in prostate cancers suggested that the cell cycle inhibitor, p27, is an important effector of growth arrest in the prostate. In the work of the last 2 years, we have shown that the cell cycle regulator, p27, mediates growth arrest by the vitamin D3 analog, EB1089. Work during final year of the grant period has addressed how processes regulating p27 are altered during prostate cancer progression. Effects of androgens and VDR activation by EB1089 on p27 function were assayed. We demonstrated that physiologic concentrations of DHT and EB1089 have synergistic effects to upregulate p27 and inhibit growth of prostate cancer cells. This work has led us to assay the effects of a combination of low dose DHT and EB1089 in pre-clinical trials using LNCaP xenografts in immunodeficient mice. Our preliminary data analysis of these in vivo studies in mouse models suggest that DHT and EB1089 causes synergistic inhibition of prostate cancer growth and can prevent tumor formation in nude mouse models in this in vivo mouse model. Data from these studies would support the inception of clinical trials of the combination of low dose DHT and EB1089 in prostate cancer patients. Unraveling the pathways whereby these steroid hormones influence the cell cycle has defines p27 as a novel target for anti-prostate cancer drugs. Moreover, our studies of p27 protein expression before and after NHT may provide a new marker to identify hormone resistant primary prostate cancers and stimulate development of novel treatment strategies.

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INTRODUCTION

Androgen interaction with the androgen receptor (AR) is important for prostate cancer cell proliferation (1, 2). The most effective therapy for prostate cancer is reduction of testosterone or of its most active metabolite, 5α-dihydrotestosterone (DHT) by androgen ablation (3). However, disease progression to an androgen insensitive state severely limits treatment efficacy. Vitamin D3 suppresses prostate cancer growth in culture and in animals (4-8). However, growth inhibition by vitamin D3 is reduced in androgen independent prostate cancer lines, suggesting a connection between the actions of vitamin D3 and androgens on the cell proliferation cycle (8-10). In prostate cancer, oncogenic events may activate peptide growth factor signaling, leading to altered cross-talk with both AR and vitamin D3 receptor (VDR) pathways. There is very limited data on mechanisms whereby androgens or vitamin D3 affect the cell cycle in prostate cells. This proposal will examine how these two steroid hormones influence key cell cycle regulators and how these mechanisms are disrupted in prostate cancer progression. The cell cycle is governed by a family of cyclin dependent kinases (cdks) which are activated by cyclin binding and inhibited by cdk inhibitors (11). Passage through G1 phase is regulated by the activities of cyclin D- and cyclin E-associated cdks. The cdk inhibitor, p27, acts in early G1 to inhibit cyclin D1/cdk4 and cyclin E/cdk2 (12). Ubiquitin proteolysis regulates p27 levels and may be triggered by phosphorylation, by changes in associated proteins or by shifts in localization (13, 14). p27 increases during differentiation induced by both vitamin D3 (15, 16) and by high dose androgen (17). The loss of p27 in primary prostate cancers (18) and the observation that p27 knockout mice develop prostatic hyperplasia (19-21)(and A. Koff unpublished results), indicate that p27 is an important negative regulator of prostate epithelial proliferation. Further, our data in LNCaP suggest that p27 mediates G1 arrest by 100 nM DHT (see Oncogene reprint, Appendix 1).

The Hypothesis and Aims of work proposed in the original and revised grant are summarized below:

We postulate that androgen ablation and vitamin D3 analogs induce prostate cell cycle arrest through inhibition of p27 phosphorylation, thereby inhibiting p27 degradation. Altered p27 regulation in prostate cancer may underlie resistance to cytostasis by androgen ablation. This will be pursued as follows. AIM 1. To identify mechanisms whereby AR and VDR activation influence the cell cycle, we will compare effects of androgen, vitamin D3 analogs and AR blockade on cell cycle regulators in steroid sensitive and insensitive prostate lines. AIM 2. To test the role of p27 phosphorylation in G1 progression, we will assay whether proteasome inhibitors have the same effect as non-steroidal androgen receptor blockers on p27 levels. We will also mutate different p27 phosphorylation sites and test their effects on p27 stability, cdk binding and inhibitory activity. AIM 3. To determine whether p27 is required for growth inhibition by androgen blockade or by vitamin D3 analogs, we will use either dominant negative p27 or p27 antisense strategies in steroid sensitive prostate lines. AIM 4. We will test whether low levels of p27 in primary prostate cancers after pre-operative androgen ablation predict for androgen resistance in vivo. AIM5 Effects of Vitamin D3 analog EB1089 and low dose androgen on growth of human prostate cancers in an invivo mouse model will be assayed (new work approved by last year's progress report).

PROGRESS REPORT BODY

The research accomplished during the period from January 2000 to January 2001 is reported below.

I. Cell cycle effects of androgen, Vitamin D3 analogs and androgen receptor blockade in steroid sensitive and insensitive prostate.

Progress: The cell lines LAPC-4 and PCa-2 proved to be unsuitable for the studies proposed. The LAPC –4 line grew poorly in 2-dimensional culture and was not androgen dependent for growth. The PCa-2 line grew very slowly and was not useful for cell cycle studies. Thus all of the studies done so far have used the LNCaP prostate cancer cell line. Expression and activities of cyclins, cdks, cdk inhibitors and their complex formation and cell cycle effects were assayed at intervals after asynchronous cell populations were treated with high dose androgen (100 nM DHT) , with the vitamin D3 analogog, EB1089 with or without low dose androgen

A) High dose (100nM) dihydrotestosterone (DHT) causes G1 cell cycle arrest in LNCaP. The following summarizes our findings. Exposure to high dose DHT inhibits population growth of the human prostate carcinoma cell line, LNCaP. To determine the mechanism of growth arrest by high dose DHT, we assayed the changes in cell cycle profile and the cell cycle regulators that mediate these effects. Treatment of asynchronously growing LNCaP cells with 100nM DHT caused a G1 arrest. The proportion of cells in S phase fell from 20% to 2%, while the G1 fraction rose from 74% to 90% by 24 hours. Loss of phosphorylation of the retinoblastoma protein was noted and cdk4 and cyclin E/cdk2 activities fell. Inhibition of these G1 cyclin dependent kinases was not due to loss of either cyclin or cdk proteins nor to increases in the cdk inhibitors $p16^{INK4A}$ and $p21^{Cip1}$. $p21^{Cip1}$ protein levels remained constant, and cyclin E-associated $p21^{Cip1}$ fell, suggesting that $p21^{Cip1}$ is not relevant to this form of cyclin E/cdk2 inhibition. Of note, total $p27^{Kip1}$ levels and cyclin E-associated $p27^{Kip1}$ increased as cells arrested and the amount of the CAK activated cdk2 bound to cyclin E decreased. $p27^{Kip1}$ immunodepletion experiments demonstrated that the DHT-mediated increase in $p27^{Kip1}$ was sufficient to fully saturate and inhibit target cyclin E/cdk2. The inhibition of cyclin E/cdk2 by $p27^{Kip1}$ contributes to G1 arrest of LNCaP following high dose DHT. $p27^{Kip1}$ may be a key effector of androgen dependent growth modulation in prostate cancer cells. **This work was published in Oncogene (see Appendix 1).**

B) The vitamin D3 analog EB1089 and low dose 3nM DHT act synergistically to arrest growth of LNCaP cells. Treatment of asynchronously growing LNCaP cells with 10^{-7} M EB1089 caused a gradual reduction in %S and an increase in the % cells in G1 (see Fig 1, Appendix 2). **All of the Figures 1-9 for this data are shown in Appendix 2.** Western analysis showed a progressive loss of cyclin A and B proteins and an increase in cdk inhibitor proteins p21, p27 and p16 (see Fig 2). This treatment had no effect on levels of cyclins E, D1, D2 nor on cdk2, cdk4 or cdk6 levels. There was a gradual loss of cyclin E- and cyclin D1-dependent kinase activities accompanying the G1 arrest of these cells (see Fig 3). IP/western analysis showed a progressive increase in the amount of cyclin E-bound p21 and p27 (see Fig 4 and 5), consistent with a role for both of these cdk inhibitors in inhibition of cyclin E-cdk2 complexes during the EB1089-induced arrest.

The combination of Vitamin D3 and a physiologic does of DHT has been shown to cause a synergistic growth arrest in prostate cancer cells (22). The vitamin D3 analogue EB1089 has the growth inhibiting properties of Vitamin D3 on prostate cancer cells, but does not cause hypercalcemia. EB1089 is currently in use in clinical trials as a neoadjuvant hormonal therapy prior

to prostatectomy in prostate cancer patients in Canada. The EB1089 has few side effects and has been well tolerated by the men in these studies. To test whether the combination of EB1089 with low, physiologic dose DHT might be effective as an anti-prostate cancer therapy, we first assayed the effects of these drugs together on the cell cycle profile of LNCaP grown in tissue culture. The combination of EB1089 and DHT caused a much more rapid and complete G1 arrest in LNCaP than did EB1089 alone (see Fig 6). Low dose DHT(3nM) did not significantly affect the cell cycle on its own (not shown). The EB1089+DHT treated cells showed a more rapid and profound effect on losses of cyclin A and cyclin B1 proteins and on the increase of p27 (Fig 7). Again, levels of cyclin E protein and the cdks were not significantly changes by either drug combination. EB1089+ DHT caused a loss of cyclin D1 by 48 hours. There was little change in p21 levels in the first 48 hours of drug treatment although EB1089 caused a significant increase in p21 levels by 4-6 days post treatment. In Figs 8 and 9, it can be seen that the inhibition of cyclin E-cdk2 activity with EB1089+DHT was much more rapid over the 48 hours of treatment than that achieved with EB1089 alone. Cyclin E immune complexes showed a 5 fold increase in the amount of associated p27 in the EB1089+DHT treated cells. Our earlier work with LNCaP (see Appendix 1) suggests that the increase in p27 binding to cyclin E-cdk2 plays an important role in the G1 arrest of LNCaP cells treated with high dose DHT. We now have data that suggests that p27 is also a key mediator of the proliferative inhibition caused by EB1089 with low dose DHT. These data strongly suggest that p27 plays a key role in this form of drug arrest and that may be an important target for the development of novel molecular based therapies for prostate cancer.

D) Effects of vitamin D3 analog EB1089 with or without low dose 3nM DHT on LNCaP tumor growth in vivo in a mouse model.

Because of the notable synergy of EB1089 and DHT in causing growth arrest of prostate cancer cells in tissue culture, we extended our studies in the final year of this grant (2000-2001) to an analysis of prostate cancer growth in an immunodeficient mouse model. A total of 40 mice were injected with DHT only, EB1089 only, DHT plus EB1089 or sham treated. The effects of these drug treatments on previously established subcutaneous LNCaP tumors was measured in these mice over approximately 4-5 months. LNCaP injected subcutaneously in a mixture of Matrigel in the CRl:nu/nu(CD-1)BR variant of nude mice, gave tumor take rates of 98%. A copy of the Animal Use Protocol that was approved by our institutional animal care committee is submitted as **Appendix 3.** All experiments were carried out in conformity with the instructions in the "Guide for Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, revised 1985). Although the statistical analysis of this data is not yet completed, our preliminary analysis suggests that treatment with EB1089 and implantation of DHT pellets in the experimental animals is more effective at inhibiting prostate cancer growth in vivo in nude mice than is EB1089 alone (See Appendix 2 Figure 10). Moreover, over the last year we also assayed whether the use of EB1089 in combination with low dose DHT (achieved by castration of the animals and implantation of DHT pellets which provide continuous stable physiologic DHT levels) was effective in preventing the development of prostate cancers when the LNCaP cells are injected subcutaneously in nude mice. A total of 80 mice were divided into 4 arms (1) low dose DHT alone 2) EB1089 alone 3) DHT plus EB1089 and 4) sham treatment controls. After 2 weeks of drug treatment, the LNCaP tumor cells were innoculated subcutaneously both flanks of the mice and the effects of drug treatment on the latency of tumor development and tumor growth were assayed over the next 6 months. The experimental work with the animals has been completed during the grant period. The analysis of tumor volumes after drug treatment requires not only measurement of the tumor size, but an analysis of the degree of necrosis within the tumor. The degree of necrosis in

the tumors has been scored by my collaborating pathologist. The final analysis of drug effects on tumor volumes will be completed in the next few months by the collaborating biostatistician.

II. Do changes in p27 phosphorylation regulate changes in p27 stability?

Progress: We have constructed 42 different p27 phosphorylation site mutant allele. These have been introduced into expression vectors for protein production in E coli, and also into a vector as a fusion with green fluorescence protein to allow immunofluorescence detection in mammalian cells. We have also got these mutant p27 cDNAs into a muristerone A inducible expression system. We have established a preliminary 2-dimensional phosphopeptide map of wt endogenous p27 protein and will be able to compare the wt pp27 phosphopeptide map with that of proteins encoded by the different phosphomutants. While this work has not matured to produce a publication, the 2 years of support and modest funding of this grant have allowed important inroads to be made into how phosphorylation regulated p27 function. This is a long term ongoing project in the lab and is now supported by a grant from the Canadian NCIC.

III. How localization of p27 regulates its protein stability

Progress: We have a paper that will be submitted to Genes and Development on how nuclear export of p27 precedes and is linked to the proteolysis of this protein. Enhanced proteolysis of the cdk inhibitor $p27^{Kip1}$ precedes and regulates the G1 to S phase transition. We show that active nuclear export of p27 is mediated by CRM1 binding in early G1. Addition of leptomycin B in mid-G1, increased p27 levels and prevented S phase entry. We identified a NES whose mutation inhibited p27/CRM1 interaction, nuclear export and p27 degradation. Exit from G0 was associated with changes in p27 phosphorylation. T187 phosphorylation of p27 preceded but was not required for CRM1 binding and export. In contrast, mutation of S10 to alanine impaired both CRM1 binding and p27 export, and increased p27 protein stability. Thus, active nuclear export of p27 is phosphorylation-dependent and a key step regulating p27 degradation.

IV. Do low levels of p27 in prostate cancers after pre-operative androgen ablation predict for androgen resistance in vivo?

Progress: The patient hospital chart review has been completed for 49 patients with prostate cancer who received neo-adjuvant hormonal therapy prior to their prostatectomy at Sunnybrook Health Sciences Centre. Chart review has been completed for 125 patients who underwent resection of their prostate cancer without prior neo-adjuvant hormonal therapy. This chart review and collection of clinical data had to be completed required prior to the retrieval of blocks and the p27 immunohistochemistry (IHC) staining of biopsy and surgically removed tumor samples. The IHC staining of p27 was completed within the time frame of the grant. We are currently waiting for the scoring of the p27 protein levels by our collaborating pathologist. The statistical analysis of the IHC results vs patient outcome will proceed when the pathologist has finished the scoring of the slides. To complete the analysis of whether low p27 in the biopsy specimen can predict for failure of neoadjuvant hormonal therapy, the number of cases analyzed must be increased. These studies will not only confirm the importance of p27 as a prognostic factor for prostate cancer, they may also indicate that low p27 levels in the tumor biopsy specimen predict for hormone independence and poor outcome. This information would be of great value in making treatment decisions for patients with prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

1. Completed studies demonstrating that the G1 arrest of the LNCaP prostate cancer line by high dose dihydrotestosterone (DHT) is mediated by the cdk inhibitor p27 (see *Oncogene* reprint appended).

2. Showed that the vitamin D3 analog, EB1089, induces G1 arrest and demonstrated roles for p21 and p27 in this arrest.

3. Demonstrated that the vitamin D3 analog, EB1089 works synergistically with low, physiologic doses of DHT to mediate G1 cell cycle arrest. The two drugs work faster than EB1089 alone, due to a more rapid induction of p27 and increase its bind to cyclin E-ckd2 and an increase to binding to cyclin E-cdk2.

4. Obtained preliminary data that EB1089 works synergistically with low dose DHT to inhbit prostate cancer growth in two different studies in immunodeficient mice. In the first study, 40 mice were treated with drugs after tumors were established and in a second study of 80 mice, drug treatment was started before mice were innoculated subcutaneously with LNCaP tumor cells and effects on tumor take and subsequent tumor growth were measured. All experimentla work is complete but the analysis of results is pending.

5. Demonstrated that nuclear export of p27 is regulated by CRM1/ran dependent mechanism and is linked to phosphorylation of p27.

6. Found nuclear export sequence for p27.

7. Mutated putative phosphorylation sites and made 40 new p27 expression vectors for recombinant protein production and expression of phosphomutant p27 in mammlian cells. Worked out a protocol for an established a 2-dimensional phosphopeptide map of wt p27.

8. Demonstrated that the use of antisense p27 oligonucleotides is not technically feasible in the LNCaP cell line. Although antisense p27 oligos prevented the increase in p27 levels following high dose DHT, the reduction in p27 protein was not sufficient to prevent its increases binding to cyclin E-cdk2 and G1 arrest by DHT in these cells (see *Oncogene* paper appended).

9. Re Aim 4 of the Grant: Chart review has been completed for 49 patients with prostate cancer who received neo-adjuvant hormonal therapy prior to their prostatic resection. Chart review has been completed for 125 patients who underwent resection of their prostate cancer without prior neo-adjuvant hormonal therapy. p27 immunohistochemistry staining of biopsy and surgically removed tumor samples has been carried out. We await the review of p27 scoring in the tumors by the collaborating pathologist.

REPORTABLE OUTCOMES

Manuscripts

Tsihlias J, Zhang W, Bhattacharya N, Flanagan M, Klotz L, Slingerland J. Involvement of $p27^{Kip1}$ in G1 arrest by high dose 5α-dihydrotestosterone in LNCaP human prostate cancer cells. Oncogene 19:670-679, 2000. (see Appendix 1)

Connor, M., Kotchetkov, R., Carious, S., Hengst, L., Melchior, F., Slingerland, M. (2000) Nuclear export of p27 is regulated by RanGTP/CRM1 binding

CONCLUSION AND FUTURE DIRECTIONS

The molecular basis of growth inhibition by androgen ablation is poorly understood. Our data in LNCaP and *in vivo* in prostate cancers (18) suggest that p27 is an important effector of growth arrest in the prostate. In the work of the last year, we have identified that the cell cycle regulator, p27, mediates growth arrest by high dose DHT (Oncogene paper appended) and also by the vitamin D3 analog, EB1089. Effects of VDR activation and androgens on p27 function were assayed. During this grant period, we have made considerable progress in developing the vectors and protocols required to clarify how phosphorylation affects p27 stability and its inhibitory function towards target cdks. Studies of how p27 phosphorylation affect its function are being continued within the context of another grant.

We have demonstrated the synergistic effect of low, physiologic concentrations of DHT and EB1089 in growth inhibition of LNCaP prostate cancer cells in tissue culture. We showed that this cell cycle arrest was mediated by the cdk inhibitors p21 and p27. This work led to our proposal to assay the effects of a combination of low dose DHT and EB1089 in pre-clinical trials using LNCaP xenografts in immunodeficient mice. Our preliminary analysis of this data suggest that low dose DHT and EB1089 do have synergistic effects to inhibit growth of LNCaP prostate tumor growth *in vivo* in nude mouse models. If these data are confirmed on final statistical analysis, these studies could lead to clinical trials in prostate cancer patients. EB1089 is already being used in clinical trials in Canada in the neo-adjuvant setting prior to prostate cancer surgery.

Unraveling the pathways whereby these steroid hormones influence the cell cycle may define novel targets for anti-prostate cancer drugs. Moreover, our studies of p27 protein expression before and after NHT may provide a new marker to identify hormone resistant primary prostate cancers and stimulate development of novel treatment strategies.

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APPENDIX 1

Oncogene (2000) 19, 670–679

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Involvement of $p27^{Kip1}$ in G1 arrest by high dose 5α-dihydrotestosterone in LNCaP human prostate cancer cells

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The cell cycle is governed by cyclin dependent kinases (cdks), which are activated by binding of cyclins, inhibited by cdk inhibitors and regulated by phosphorylation and dephosphorylation. Exposure to high dose dihydrotestosterone (DHT) inhibits population growth of the human prostate carcinoma cell line, LNCaP. To determine the mechanism of growth arrest by high dose DHT, we assayed the changes in cell cycle profile and the cell cycle regulators that mediate these effects. Treatment of asynchronously growing LNCaP cells with 100 nM DHT caused a G1 arrest. The proportion of cells in S phase fell from 22 to 2%, while the G1 fraction rose from 74 to 92% by 24 h. Loss of phosphorylation of the retinoblastoma protein was noted and cdk4 and cyclin E/cdk2 activities fell. Inhibition of these G1 cyclin dependent kinases was not due to loss of either cyclin or cdk proteins nor to increases in the cdk inhibitors $p16^{INK4A}$ and $p21^{Cip1}$. $p21^{Cip1}$ protein levels remained constant, and cyclin E-associated $p21^{Cip1}$ fell, suggesting that $p21^{Cip1}$ is not relevant to this form of cyclin E/cdk2 inhibition. Of note, total $p27^{Kip1}$ levels and cyclin E-associated $p27^{Kip1}$ increased as cells arrested and the amount of the CAK activated cdk2 bound to cyclin E decreased. $p27^{Kip1}$ immunodepletion experiments demonstrated that the DHT-mediated increase in $p27^{Kip1}$ was sufficient to fully saturate and inhibit target cyclin E/cdk2. The inhibition of cyclin E/cdk2 by $p27^{Kip1}$ contributes to G1 arrest of LNCaP following high dose DHT. $p27^{Kip1}$ may be a key effector of androgen dependent growth modulation in prostate cancer cells. *Oncogene* (2000) **19**, 670–679.

Keywords: p27; cell cycle; prostate cancer; androgen; cyclin E/cdk2

Introduction

Androgen interaction with the androgen receptor (AR) is important for growth and development of both the normal prostate and of prostate cancer (Hakimi *et al.*, 1996). At present, the most effective therapy for prostate cancer is reduction of testosterone or of its most active metabolite, 5α-dihydrotestosterone (DHT) by different treatments collectively referred to as androgen ablation (Catalona, 1994). However, tumor progression to an androgen insensitive state severely limits the efficacy of these treatments. We have investigated how this steroid pathway influences key cell cycle regulators in the androgen sensitive, human prostate cancer cell line, LNCaP. An understanding of how cell cycle progression is influenced by androgen pathways, and how these mechanisms are disrupted in prostate cancer progression, may lead ultimately to new methods of achieving cytostasis in hormone resistant prostate cancers.

Progression through the cell cycle is governed by a family of cyclin dependent kinases (cdks), whose activity is regulated by phosphorylation (Solomon, 1993), activated by binding of cyclins (Morgan, 1995; Sherr, 1994) and inhibited by the cdk inhibitors (Reed *et al.*, 1994; Sherr and Roberts, 1995). The cdks regulate biochemical pathways, or checkpoints, which integrate mitogenic and growth inhibitory signals, monitor chromosome integrity, and coordinate cell cycle transitions (Hartwell, 1992; Murray, 1992). Passage through G1 into S phase is regulated by the activities of cyclin D-, cyclin E-, and cyclin A-associated kinases. Cyclin B-dependent kinases regulate the G2/M transition.

Two families of cdk inhibitors mediate cell cycle arrest following growth inhibitory stimuli (Sherr and Roberts, 1995, 1999). The inhibitor of cdk4, INK4, family members $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$ bind cdk4 and cdk6 specifically and inhibit cyclin D binding. Members of the KIP or kinase inhibitor protein family, which include $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, bind and inhibit target cyclin/cdk complexes. The cdk inhibitor, p27, acts during G0 and the early G1 phase of the cell cycle to inhibit G1 cyclin/cdk complexes (Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994; Slingerland *et al.*, 1994; Hengst *et al.*, 1994). In many cell types, p27 is essential for quiescence. Antisense p27 inhibits exit from the cell cycle following serum withdrawal (Coats *et al.*, 1996; Rivard *et al.*, 1996) and antisense p27 stimulates estradiol depleted quiescent MCF-7 breast cancer cells to re-enter cell cycle (Cariou, Donovan and Slingerland, in preparation). p27 levels are regulated by post-transcriptional mechanisms including both translation and proteolysis (Hengst and Reed, 1996; Pagano *et al.*, 1995; Millard *et al.*, 1997). In human cell lines, phosphorylation of p27 is maximal in G1, just prior to the drop in p27 protein that occurs at S phase (Pagano *et al.*, 1996). Phosphorylation of p27 triggers its proteolytic degradation (Vlach *et al.*, 1997; Sheaff *et*

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Received 5 February 1999; revised 8 November 1999; accepted 23 November 1999

al., 1997; Montagnoli *et al.*, 1999; Tsvetkov *et al.*, 1999), as is the case for the yeast cdk inhibitor Sic1 (Schneider *et al.*, 1996; Tyers, 1996).

p27 increases during differentiation in many cell types, including differentiation induced by vitamin D3 (Hengst and Reed, 1996; Wang *et al.*, 1996) and by androgen in the prostatic epithelium (Chen *et al.*, 1996). p27 knockout cells manifest altered differentiation programs (Casaccia-Bonnefil *et al.*, 1997). Indeed, p27 knockout mice develop multiple organ hyperplasia (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996), including prostatic hyperplasia (Cordon-Cardo *et al.*, 1998), underlining the importance of p27 as an inhibitor of prostate cell proliferation.

Increasing evidence suggests that the cyclins, cdks and cdk inhibitors are either themselves targets for genetic change in cancer or are disrupted secondarily by other oncogenic events (Hunter and Pines, 1994). Since they oppose mitogenic stimuli, the cdk inhibitors are good candidates as tumor suppressors. Although genetic changes in the *p16* gene support a tumor suppressor (TS) role for this inhibitor in cancers (Bates and Peters, 1995), mutations in *p27* have been identified only rarely (Kawamata *et al.*, 1995; Pietenpol *et al.*, 1995; Ponce-Castaneda *et al.*, 1995). A reduction in p27 protein could contribute to resistance to growth inhibitory factors, deregulation of cell proliferation, and oncogenic change. Although normal human epithelia of the breast, prostate, lung, and colon express high levels of p27 protein, this protein is frequently reduced in primary carcinomas at these sites (reviewed in Cariou *et al.*, 1998). The reduction of p27 protein is of prognostic importance in these cancers and correlates with an aggressive tumor phenotype *in vivo* (Catzavelos *et al.*, 1997; Loda *et al.*, 1997; Porter *et al.*, 1997; Tan *et al.*, 1997; Mori *et al.*, 1997; Esposito *et al.*, 1997; Tsihlias *et al.*, 1998). Enhanced proteolytic degradation may underlie the loss of p27 in tumor cells (Catzavelos *et al.*, 1997; Loda *et al.*, 1997; Esposito *et al.*, 1997). Thus, changes in the post-translational mechanisms which target p27 for degradation, may be germane to oncogenesis and/or tumor progression.

In a study of p27 immunostaining in primary prostate cancer, we found that loss of p27 protein was an independent prognostic factor, predicting reduced time to recurrence following radical prostatectomy ($P=0.047$, risk ratio 2.08) (Tsihlias *et al.*, 1998). In a small subset of patients, androgen ablation therapy prior to tumor removal appeared to increase p27 protein, raising the possibility that androgens may modulate p27 degradation pathways *in vivo*. In the present study, the effect of high dose androgen on cell cycle progression was investigated in a model of human prostate cancer, the LNCaP cell line.

LNCaP is an androgen-sensitive human prostate cancer cell line derived from a metastasis to a supraclavicular lymph node (Horoszewicz *et al.*, 1983). The line has an aneuploid human male karyotype, and is tumorigenic when implanted into nude mice. The potent androgen, 5α-dihydrotestosterone (DHT), modulates LNCaP proliferation in a well-characterized fashion. DHT stimulates proliferation at concentrations between 0.1 and 1.0 nM DHT and proliferation is inhibited at DHT concentrations below 0.1 nM or above 10 nM DHT. Androgens have also been shown to mediate biphasic growth response in the prostate *in vivo*. Low dose androgen administered to castrate rats will reverse prostate atrophy and induce proliferation in the prostate gland. Higher doses of androgen cause growth arrest by inducing differentiation of prostate epithelial cells (Chen *et al.*, 1996). Because our study of primary tumors suggested that p27 regulation may be importantly altered in human prostate cancers, we wished to explore further how androgen may modulate the cell cycle and p27 function in the prostate cancer line, LNCaP.

Results

100 nM dihydrotestosterone causes G1 arrest of LNCaP

Treatment of asynchronously growing LNCaP cells with 100 nM DHT for 24 h induced a G1 arrest. The proportion of cells in G1 went from 70%, in the asynchronously growing untreated cells, to 92% at

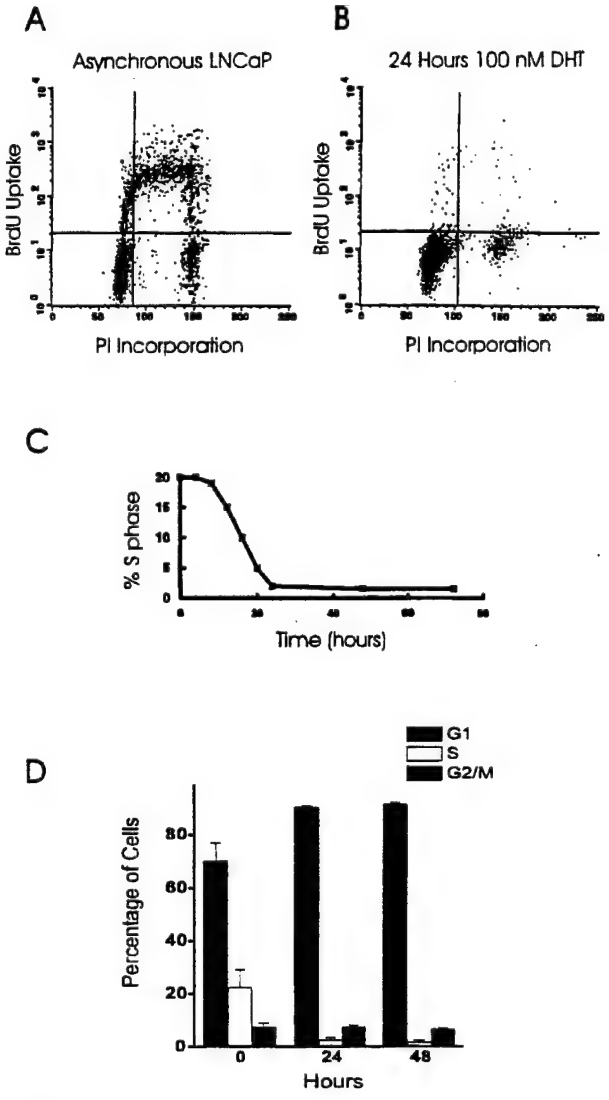

Figure 1 FACS analysis of LNCaP treated with 100 nM dihydrotestosterone. (**a**) Dot plot showing relative uptake of BrdU and PI in asynchronously growing LNCaP. (**b**) Decreased BrdU uptake indicating arrest of DNA synthesis after 24 h treatment with 100 nM DHT. (**c** and **d**) Cells were harvested and prepared for FACS analysis at various intervals after addition of 100 nM DHT to culture media. G1 arrest was maximal at 24 h. Results shown in (**d**) are the average of three separate experiments

maximal arrest by 48 h. Cells in S phase fell from 22% of the untreated population to 2% within 24 h (Figure 1). A sub-G1 population was not observed. No significant growth arrest was observed when cells were cultured in media supplemented with 1 and 10 nM DHT (data not shown).

The cell cycle arrest persisted for up to 96 h in media supplemented with DHT. This DHT-mediated cell cycle arrest was not accompanied by cytotoxicity, based on morphological appearance and the use of Trypan blue exclusion staining. The proportion of cells that failed to exclude Trypan blue was similar in treated and untreated cells. Treatment of LNCaP cells with 50 nM DHT also caused a G1 arrest within 48 h. To determine whether high dose DHT was inducing a form of irreversible differentiation, cells were cultured for 48 h in 50 and 100 nM DHT and then transferred to media without additional DHT for a further 24 h. Upon removal of supplemental DHT from the culture media, cells underwent a prompt release from G1 arrest, showing an asynchronous cell cycle profile within 24 h (S phase fraction 20%) (Table 1).

To determine whether the effects of 100 nM DHT on prostate cancer cell proliferation could occur in the absence of the AR and AR signaling, two steroid-independent prostate cancer lines, PC-3 (Kaighn *et al.*, 1979) and DU145 (Stone *et al.*, 1978) were treated with 100 nM DHT. Neither the PC-3 nor the DU145 prostate cancer cell lines express detectable AR (Kaighn *et al.*, 1979; Stone *et al.*, 1978). The addition of 100 nM DHT to the culture medium had no effect on either PC-3 or DU145 cell proliferation (data not shown). Treatment of LNCaP cells with 100 nM DHT for 48 h led to an increased secretion of prostate specific antigen as determined by Hybritech assay on culture media. PSA concentration (ng/ml) in conditioned media from cells with/without 100 nM DHT was 383.5/167.5 and 443.1/340.3 on two separate occasions. Although the results were not normalized for cell number, cells were initially plated equally. After 48 h, the number of cells on DHT treated plates would be less than that on untreated plates. Thus, relative cellular production of secreted PSA was increased by exposure to 100 nM DHT. A Western blot did not demonstrate any difference in cellular PSA levels as cells underwent arrest (data not shown).

DHT mediated G1 arrest is accompanied by accumulation of nuclear p27 protein

Immunohistochemistry performed on asynchronous populations of LNCaP cells demonstrated a heterogeneous nuclear immunoreactivity for p27. Cells arrested in G1 after treatment with 100 nM DHT for 48 h demonstrated uniformly strong nuclear staining for p27 protein (Figure 2). Controls reacted with secondary antibody alone did not show any significant nuclear staining. Pre-adsorption of the primary antibody with blocking peptide abolished the nuclear staining (data not shown).

Western analysis at intervals after treatment with 100 nM DHT showed loss of phosphorylation of the retinoblastoma protein (pRb) within 16 h, with predominantly hypophosphorylated pRb detected at 48 h. There was no appreciable change in the protein levels of cdk2, cdk4, and cdk6, cyclins D1 and E, or the cdk inhibitors $p16^{INK4A}$ and $p21^{Cip1}$. Levels of cyclins A and B1 fell significantly. This is consistent with the DHT-mediated inhibition of entrance into S and G2/M phases, where peak expression of these latter two proteins is known to occur. An increase in p27 protein levels was appreciable within 4 h after addition of DHT, with peak levels fourfold above baseline detected by 48 h, as determined by densitometry (Figure 3).

Table 1 Cell cycle profiles of LNCaP

Culture conditions	*%G1*	*%S*	*%G2/M*
Asynchronously growing cells	76	19	5
100 nM DHT 48 h	93	2	5
100 nM DHT 48 h then no DHT 24 h	74	19	7
50 nM DHT 48 h	93	2	5
50 nM DHT 48 h then no DHT 24 h	72	20	8

Asynchronously growing cells were cultured in RPMI 1640 plus 5% FBS with either 50 or 100 nM 5α-dihydrotestosterone (DHT) for 48 h. Cells were then either collected for FACS analysis or washed and fresh media without supplemental DHT was added to plates for a further 24 h prior to FACS analysis

The increase in p27 saturates and inhibits cyclin E/cdk2 in DHT-arrested LNCaP

Inhibition of cyclin E/cdk2 activity was notable within 12 h after the addition of 100 nM DHT to the asynchronously growing cells, with minimal activity detected at 24 h (Figure 4b). To further investigate the mechanism of inhibition of cyclin E/cdk2, these complexes were examined by immunoprecipitation of cyclin E followed by Western blotting to detect associated proteins. No dissociation of cyclin E/cdk2 complexes was observed, however, the amount of CAK-activated threonine 160 phosphorylated cdk2 bound to cyclin E decreased. Phosphorylation by cdk activating kinase (CAK) shifts cdk2 to its faster mobility form on SDS–PAGE (Gu *et al.*, 1992). The amount of cyclin E-associated p21 fell, suggesting that p21 is not relevant to this form of cyclin E/cdk2 inhibition. The amount of cyclin E-associated p27 increased 3–4-fold, by densitometry, as the cells entered G1 arrest (Figure 4a). In other cellular contexts, such an increase in p27 has been shown to saturate cellular cyclin E/cdk2 (Reynisdottir *et al.*, 1995).

To determine whether this increase in p27 was sufficient to saturate target cyclin E/cdk2 and induce cell cycle arrest, p27 was immunodepleted by three serial immunoprecipitations from both asynchronously growing cell lysates and from DHT-arrested cell lysates. Cyclin E immune complexes were examined before and after p27-immunodepletion. p27 immunodepletion caused a significant reduction of cyclin E protein from asynchronously growing cell lysates (Figure 4c). This reflects the substantial proportion of G1 phase cells in an asynchronous population of LNCaP. In spite of a significant reduction in the level of cyclin E, the remaining cyclin E immune complexes showed almost the same kinase activity after p27 immunodepletion as before (Figure 4c, left panel). This is consistent with most p27-bound cyclin E complexes having minimal kinase activity. There was virtually no detectable cyclin E remaining after p27 immunodepletion of DHT-arrested cell lysates. Thus, the increase in p27 by DHT treatment was sufficient to saturate and inhibit the cellular cyclin E/cdk2 complexes.

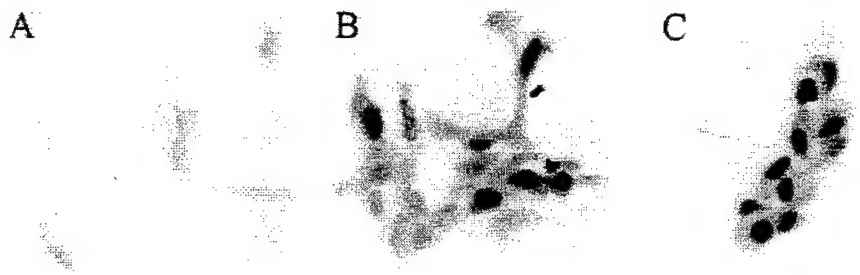

Figure 2 p27 nuclear staining of LNCaP cells in tissue culture. Cells were cultured on poly-L-lysine coated glass slides, fixed and stained for p27 as described in the Materials and methods section. (**a**) Control sample obtained by staining with secondary antibody alone. (**b**) Asynchronously growing cells. (**c**) Cells arrested by treatment with 100 nM DHT show uniform strong nuclear p27 staining

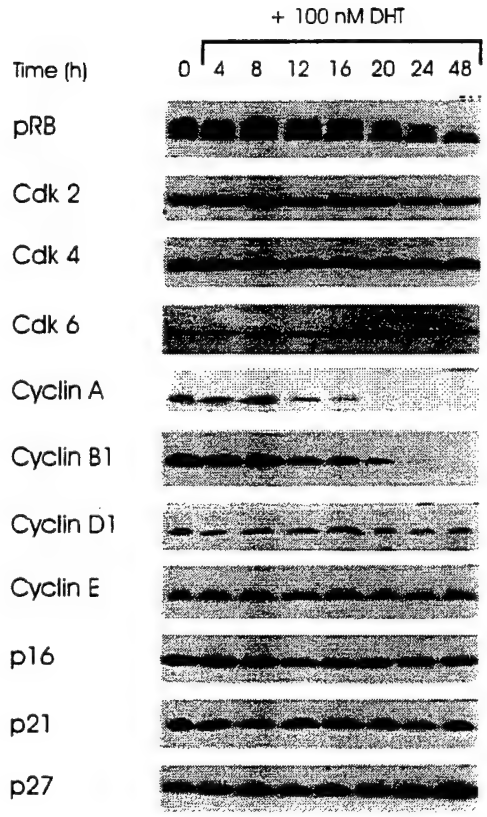

Figure 3 Cell cycle regulators during G1 arrest by DHT. Cells were harvested at indicated time intervals after addition of 100 nM DHT to culture media. Lysates were prepared and resolved using 17.5% SDS–PAGE. Proteins were detected by immunoblotting with the indicated antibodies

Antisense p27 failed to prevent the increased binding of p27 to cyclin E after high dose DHT

Antisense p27 oligonucleotides (ASp27) were transfected into asynchronously growing LNCaP cells. Six hours post transfection, 100 nM DHT was added to control (lipid alone), mismatch oligonucleotide (MSp27) and ASp27 transfected cells. In both oligonucleotide-transfected and non-transfected controls, 100 nM DHT induced G1 arrest. MSp27 and control cells showed the same increase in p27 by DHT as did LNCaP treated with 100 nM DHT alone. ASp27 prevented the fourfold rise in p27 protein following DHT treatment. p27 levels in the cells treated with ASp27 and DHT together were similar to untreated asynchronously growing LNCaP. However, when cyclin E immune complexes were analysed, ASp27 treated cells still showed an increase in the binding of p27 to cyclin E complexes following 100 nM DHT, in spite of the modest reduction in total p27 levels. ASp27 treatment did not reduce total p27 protein levels to a sufficient degree in LNCaP to prevent the DHT-mediated increased association of p27 with its target kinase cyclin E/cdk2 (data not shown).

Inhibition of cyclin D-bound cdk4 by high dose DHT

The cyclin D-associated kinases also function in G1 to regulate the transition into S phase. To assay the effects of 100 nM DHT on these complexes, cdk4 was immunoprecipitated and kinase activity assayed at various intervals during the arrest of the LNCaP cells. Cdk4 activity was strongly inhibited by treatment with 100 nM DHT (Figure 5b). Immunoprecipitation of cdk4 and its associated complexes did not demonstrate any dissociation of cyclin D1 from cdk4. The level of cdk4-bound p16 remained unchanged. There was a rise in p15 association with cdk4 between 0–12 h after addition of DHT, which persisted to 24 h. Between 24–48 h, there was no further reduction in the per cent S phase cells, and cdk4-bound p15 actually decreased slightly by 48 h, possibly reflecting progression into a quiescent state. The levels of p21 and p27 bound to cdk4 decreased as cells underwent arrest.

Discussion

The LNCaP cell line is the most commonly studied model of androgen-sensitive prostate cancer. This cell line shows a characteristic bell shaped growth curve in response to increasing concentrations of androgen in the growth media (Horoszewicz *et al*., 1983; Lee *et al*., 1995; Kim *et al*., 1996). Although the dose-dependent differences in the effects androgen on cell numbers have been known for nearly two decades, the mechanism of the growth inhibition by high dose androgen was poorly understood. Androgens are required for normal growth and differentiation in prostate epithelial cells. Chen *et al*. (1996) studied the effects of androgen on the cell cycle of normal prostate *in vivo* in rats. Castration resulted in atrophy of the gland due to

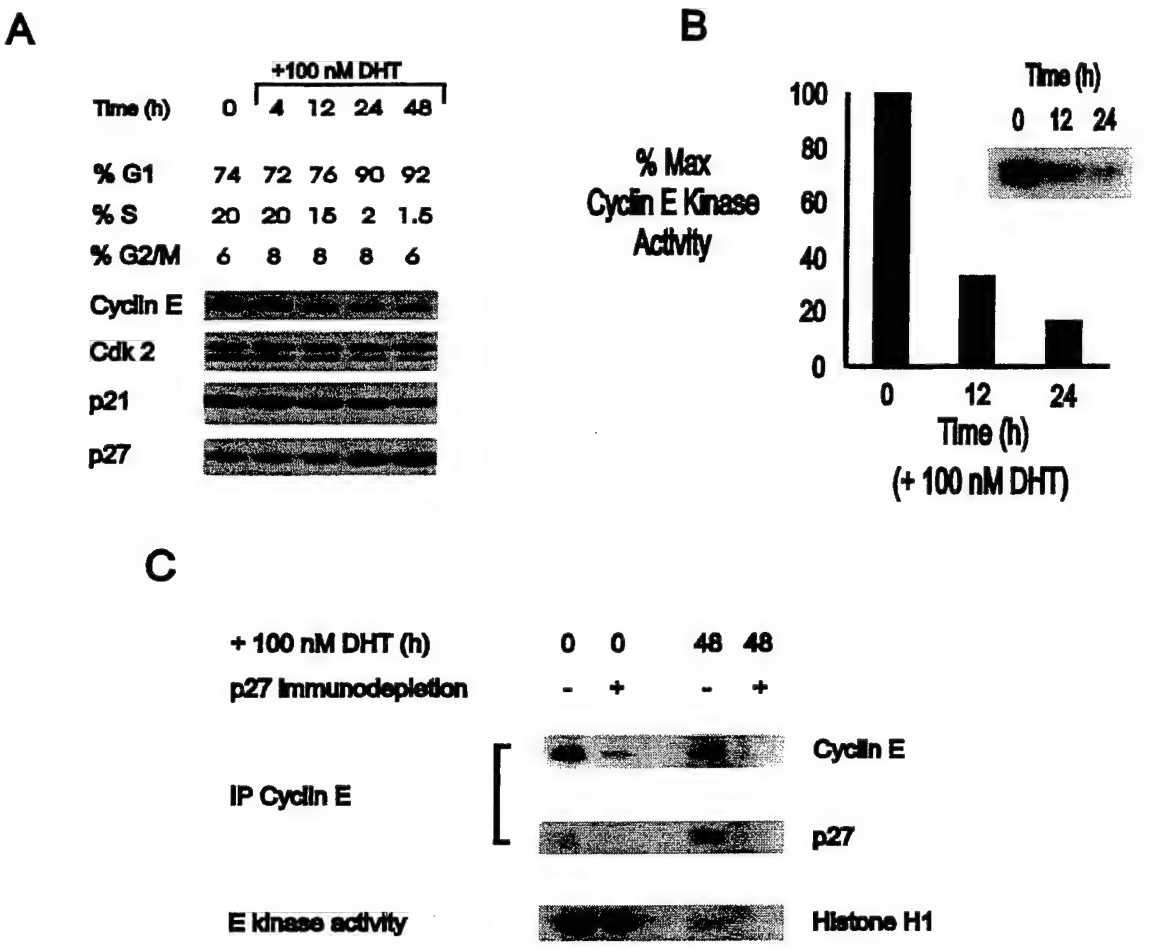

Figure 4 p27 saturates cyclin E/cdk2 complexes and inhibits this kinase following treatment of asynchronously growing LNCaP with 100 nM DHT. (**a**) Cyclin E immune complexes. Cells were recovered at intervals after addition of DHT to culture media. DNA profiles were determined by FACS analysis, and the percentage of cells in G1, S, and G2/M is indicated for each time point. Cyclin E was immunoprecipitated, complexes resolved by SDS–PAGE and immunoblots were reacted with antibodies to detect associated proteins. (**b**) Cyclin E/cdk2 Kinase Activity. Cyclin E was immunoprecipitated from lysates collected in (**a**) above and associated kinase activities were assayed. Reaction products were resolved by SDS–PAGE, and gels were dried and exposed to film. Radioactivity in the Histone H1 substrate was quantitated by PhosphorImager and expressed as a percentage of maximum activity after subtraction of background from control immunoprecipitates. (**c**) p27 immunodepletion experiments. p27 was serially immunodepleted from lysates collected at either 0 or 48 h after addition of 100 nM DHT to media. Cyclin E-associated proteins and cyclin E-associated kinase activities were assayed before and after p27 immunodepletion from the T=0 and T=48 h samples

apoptosis and arrest of surviving cells. Re-administration of testosterone induced transient epithelial cell proliferation followed by decreased cellular proliferation and an increase in p27. It was postulated that the increased p27 was playing a role to inhibit proliferation as regenerating prostatic tissue underwent steroid-mediated differentiation. The paradoxical effects of androgen stimulation in LNCaP (growth stimulation at low concentrations of androgen, growth inhibition with increased PSA secretion at high concentrations) may represent a normal physiological mechanism that has been retained by this tumor cell line.

Several lines of evidence suggest that the G1 arrest by high dose DHT is an AR mediated effect. In our study, as in others (Lee *et al*., 1995), high dose DHT induced an increase in PSA secretion by the LNCaP cells. PSA is a serine protease that is normally secreted by the prostatic epithelium. We demonstrated higher PSA production in conditioned media from cells arrested after 48 h in 100 nM DHT than in cells without added androgen. This result and our observation that cellular PSA levels were not increased by Western analysis are consistent with a DHT-induced increase in secretion of PSA. Treatment of AR-negative PC-3 cells and DU145 cells with 100 nM DHT affected neither cellular proliferation nor PSA secretion (not shown). Furthermore, PC-3 cells that have been genetically engineered to express the AR show growth arrest in response to androgen treatment (E Brown, personal communication).

The present study demonstrates that the decreased rate of growth for LNCaP at high doses of DHT is due to G1 arrest and not to cell death. Growth inhibition with G1 arrest was demonstrated at doses of 50 and 100 nM DHT, whereas, no significant growth arrest was observed with doses between 1–10 nM DHT. We saw no morphological evidence of cell toxicity and Trypan blue exclusion did not differ between treated and untreated cells. Furthermore, the G1 arrest appeared fully reversible on removal of the high dose DHT at 48 h. The longest treatment interval was 96 h, with maximal arrest noted at 24 h. Whether exposure to 100 nM DHT longer than 96 h might induce an irreversible G1 arrest is not known.

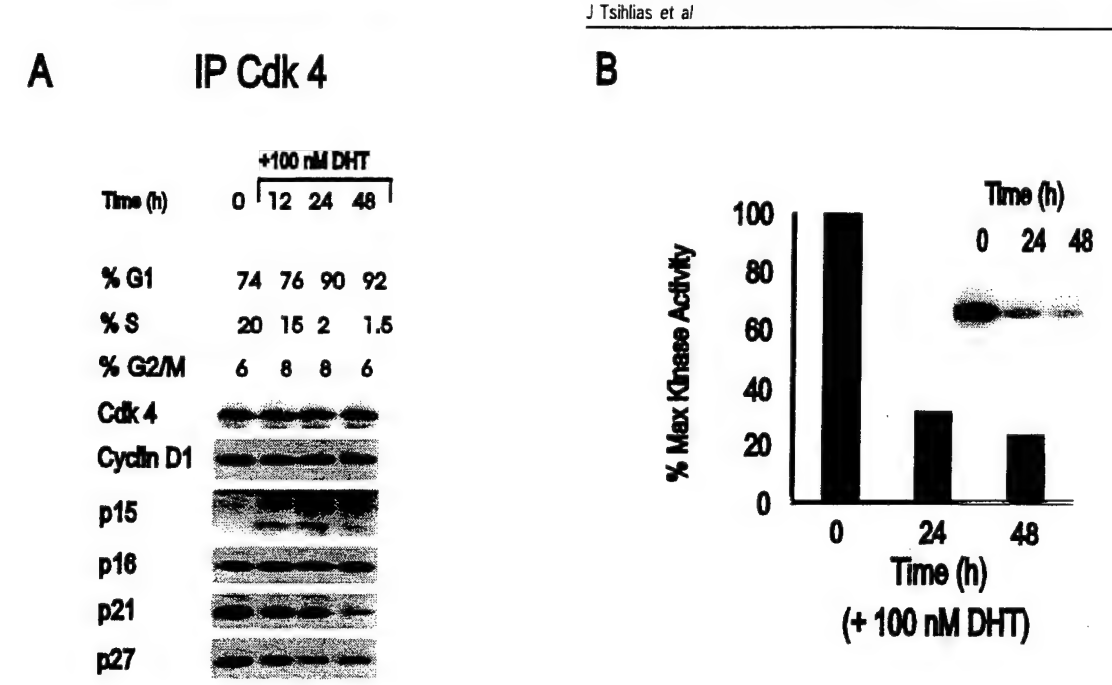

Figure 5 Inhibition of Cdk4 activity during DHT-mediated G_1 arrest. Asynchronous LNCaP cultures were treated with 100 nM DHT and cells were recovered for FACS analysis and preparation of protein lysates. (a) Cdk4 complexes. Cdk4 was immunoprecipitated and complexes were resolved and immunoblotted as for Figure 4a. (b) Cdk4 kinase assay. Cdk4 was immunoprecipitated and kinase activity was assayed as in Materials and methods

High dose DHT caused a loss of pRB phosphorylation as has been observed in other forms of G1 arrest (Laiho *et al.*, 1990; Sandhu *et al.*, 1997). Hypophosphorylated pRB binds and inactivates E2F, thereby inhibiting the function of that transcription factor, and precluding transcription of genes required for S phase entry (DeGregori *et al.*, 1995). Phosphorylation of pRB leads to its dissociation from E2F, and to E2F activation (Chellappan *et al.*, 1991). The *pRB* gene is wild type in LNCaP (Peehl, 1994). Since both cyclin D-associated kinases and cyclin E/cdk2 activities contribute to pRB phosphorylation (Sherr and Roberts, 1995), the effects of DHT on these cyclin/cdks were assayed.

Cyclin E-dependent kinase activity fell as cells underwent arrest. This was accompanied by a fourfold overall increase in p27 protein. More importantly, the increase in p27 protein was sufficient to fully saturate cyclin E/cdk2 complexes. p27-immunodepletion demonstrated that essentially all of the cellular cyclin E was bound to p27 in these DHT-arrested cells. This supports the conclusion that the increase in p27 contributes causally to cyclin E/cdk2 inhibition and to the G1 arrest observed. In other systems, a similar increase in p27 levels has been shown to be sufficient to saturate and inactivate cyclin E/cdk2 (Reynisdottir *et al.*, 1995). The fact that cyclin E-associated p21 fell as cells arrested, suggests that p21 is not likely to be involved in inhibition of this kinase.

ASp27 treatment prevented the fourfold increase in p27 levels following exposure to 100 nM DHT. However, ASp27 failed to prevent DHT-mediated G1 arrest. In spite of the modest reduction in p27 protein levels, the increased association of p27 with cyclin E/cdk2 complexes was intact in ASp27 treated cells arrested by 100 nM DHT. This suggests that factors that lead to an increased affinity of binding of p27 to cyclin E/cdk2 following DHT treatment may be more important to the inhibition of this kinase than the increase in p27 protein levels alone.

Cdk activating kinase (CAK) mediated phosphorylation of cdk2 on threonine 160 is required for catalytic activity (Gu *et al.*, 1992). In this study, cyclin E-bound cdk2 showed a progressive loss of CAK activation, with accumulation of the slower mobility form of cyclin E-bound cdk2. Kato *et al.* (1994) have demonstrated that p27 can inhibit CAK activation of cyclin D-associated cdk4 during cyclic AMP mediated G1 arrest in macrophages. Increased binding of p27 to cyclin E/cdk2 may modulate the conformation of these complexes and block the access of CAK to its catalytic sites on cdk2, thereby preventing the activating phosphorylation of cdk2. A similar loss of CAK activation of cyclin E-bound cdk2 has been demonstrated in TGF-β arrested cells (Koff *et al.*, 1993; Slingerland *et al.*, 1994).

Cdk4 kinase activity was also inhibited by 100 nM DHT. p16, p21, and p27 are not likely involved in the inhibition of this kinase, since p16 association with cdk4 remained constant and dissociation of p21 and p27 from cdk4 was observed. p15 binding to cdk4 was increased at 12 h and remained elevated until 24 h. The accumulation of p15 may facilitate dissociation of p21 and p27 from cdk4 complexes. Versions of this model of action for p15 have been suggested previously in G1 arrest due to TGF-β (Reynisdottir *et al.*, 1995; Sandhu *et al.*, 1997). It is also possible that the affinity of p27 for cyclin E/cdk2 is actively regulated as suggested by Sheaff *et al.* (1997), and this may be independent of the effects of p15 on cyclin D/cdk4 complexes (Sandhu *et al.*, 1997).

The INK4 family of cdk inhibitors have been shown to inactivate their target kinases by binding to them and displacing the associated cyclin (Parry *et al.*, 1995;

Sandhu *et al.*, 1997). Although we did not see cyclin D1 dissociation from cdk4 as kinase activity diminished, it is possible that p15 may have displaced cyclin D2 or D3. However, we were unable to detect cyclins D2 or D3 in cdk4 complexes using commercially available antibodies. Clearly, there may be more than one mechanism contributing to DHT-mediated G1 arrest. Although p27 appears to be important in the inhibition of cyclin E/cdk2, other mechanisms, including p15, may act to inhibit the D-type cyclin/cdks.

Since 1941, when Huggins and Hodges discovered that prostate cancer undergoes regression in response to castration, there have been few major advances in the management of metastatic prostate cancer (Huggins *et al.*, 1941). Leutinizing hormone releasing hormone (LHRH) analogs that induce castrate levels of testosterone have obviated the need for castration (Peeling, 1989). In spite of advances in the hormonal therapy of prostate cancers, progression to a hormone independent state invariably occurs. Thus, there is clearly a need for development of novel treatment approaches for advanced prostate cancer. A greater understanding of how cell cycle regulators respond to androgenic stimuli in normal and malignant prostatic epithelial cells could lead to the identification of new targets for therapy. The present study suggests that the increased levels of p27, and its binding and inhibition of cyclin E/cdk2 contribute to G1 arrest of LNCaP human prostate cancer cells in response to high doses of DHT.

Several other reports suggest a role for p27 as a mediator of G1 arrest in prostate cancer tissue culture models. G1 arrest of prostate cancer line DU145 induced by EGF receptor blockade involves upregulation of p27 protein and corresponding inhibition of cyclin E/cdk2 activity (Peng *et al.*, 1996; Zi *et al.*, 1998). A subline of LNCaP developed by continuous culture in the absence of androgen is growth inhibited by low doses of androgen. In this LNCaP subline, loss of cdk2 activity was also correlated with accumulation of p27 (Kokontis *et al.*, 1998). In an androgen dependent mouse mammary carcinoma cell line, SC-3, G1 arrest upon androgen withdrawal is associated with a similar upregulation of p27 and the binding and inhibition of cdk2 by p27 (Menjo *et al.*, 1998). In these different tissue culture models, androgen mediates different effects on proliferation: in the one case, androgen induces growth arrest, whereas in the other, androgen withdrawal has this same effect. Interestingly, in each case, a similar mechanism of G1 arrest appears to be invoked, with upregulation of p27 leading to inhibition of cyclin E/cdk2 activity. Since the growth of prostate cancers *in vivo* can initially be restricted by anti-androgens, it would be of interest to determine whether p27 is up-regulated following androgen depletion of steroid-dependent prostate cancer.

Increasing data from studies of p27 *in vivo*, in human prostate cancers, suggest a role for this KIP in prostate epithelial cell growth. Several recent reports indicate a prognostic role for p27 in prostate cancer. Low or undetectable levels of p27 protein in primary prostate carcinoma have been shown to correlate with increased proliferative index (Guo *et al.*, 1997), increasing tumor grade (Tsihlias *et al.*, 1998; Yang *et al.*, 1998; Guo *et al.*, 1997; Cote *et al.*, 1998) shorter disease-free survival (Tsihlias *et al.*, 1998; Yang *et al.*, 1998; Cote *et al.*, 1998; Cordon-Cardo *et al.*, 1998), and decreased overall survival (Cote *et al.*, 1998). In our study of the prognostic value of p27 in human prostate cancer, strong p27 staining was uniformly seen in benign prostatic epithelial components in all tumor sections. p27 staining was variable in prostatic intraepithelial neoplasia and reduced in most prostate cancers. A small subset of tumors, treated with pre-operative androgen ablation therapy prior to radical prostatectomy, tended to show higher expression of p27 protein than that in untreated cases. The few tumors that showed a persistence of low p27 staining (less than 25% of tumor nuclei positive) after androgen ablation therapy had the worst prognosis (Tsihlias *et al.*, 1998). In their study of p27 expression in prostate cancer, Cordon-Cardo *et al.* (1998) found low or absent p27 in androgen-independent metastatic prostate cancers. These authors also raised the possibility that mechanisms leading to accelerated p27 degradation may contribute to the development of metastases and/or progression of prostate cancer to the androgen-independent state. Taken together, both studies of p27 in prostate cancer cell lines and in primary tumors raise the hypothesis that changes in pathways that regulate p27 levels may contribute to tumor progression and to growth modulation of human prostate cancer cells in response to various androgenic stimuli.

Materials and methods

Cell culture

The fast growing strain of LNCaP, LNCaP-FGC (Berns *et al.*, 1986), and cell lines PC-3 and DU145 were purchased from ATCC. Cells were grown in RPMI 1640 culture media plus 5% FBS without phenol red. All experiments were performed using LNCaP passages 25 to 40. Cells were grown to 80% confluence in 10 cm tissue culture plates and split 1:6. Cell populations growing asynchronously were then treated with variable concentrations (0.1–100 nM) of 5α-dihydrotestosterone (Sigma Laboratories, St. Louis, MO, USA) or ethanol vehicle (0.1% and 1.0%) alone as a control. Cells were treated for up to 96 h, and the media changed every 48 h. As controls, the AR-negative prostate cancer lines PC-3 and DU145 were cultured in DMEM supplemented with 5% FBS without phenol red. Asynchronously growing PC-3 or DU145 cells were treated with 100 nM DHT for 48 h prior to recovery for flow cytometry and protein analysis.

Determination of PSA secretion

Cells were cultured on 10 cm plates in 10 ml media. At 30% confluence, cells were washed twice with PBS and then cultured with fresh medium with or without addition of 100 nM DHT. Conditioned media were collected at 48 h and PSA concentration determined using the Hybritech assay.

Immunohistochemistry

LNCaP cells were plated on poly-L-lysine coated glass slides and cultured for 24 h. The media was then supplemented with 100 nM DHT and cells cultured for a further 48 h. Cells were then washed in PBS, fixed in 4% paraformaldehyde containing 0.2% Triton X-100 for 10 min at room temperature. Slides were then blocked with 3% hydrogen peroxide in methanol followed by normal horse serum (10% solution) and then incubated overnight at 4°C with anti-p27 monoclonal antibody (Transduction Laboratories, Lexington,

KY, USA) diluted 1:1000 (0.25 μg/ml) in PBS. Slides were then reacted with biotin-labeled anti-mouse IgG and incubated with preformed avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Metal-enhanced diaminobenzidine substrate (Pierce, Rockford, IL, USA) was then added in the presence of horseradish peroxidase. Cells were photographed using a Wilovert microscope equipped with a Wild Leitz MPS 52 photocamera (Wild Leitz Ltd., Heerbrugg, Switzerland).

Flow cytometric analysis

Cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdU) for 2 h at intervals after addition of DHT to asynchronously growing cells. Cells were then harvested, fixed with 70% ethanol, treated with 0.1 N HCl, and heated for 10 min at 95°C to expose labeled DNA. Cells were then stained with anti-BrdU-conjugated FITC (Becton Dickinson) and counterstained with propidium iodide. Cell cycle analysis was carried out on a Becton Dickinson FACScan, using Cell Quest software.

Immunoblotting

Cells were lysed in ice cold NP-40 lysis buffer (0.1% NP-40, 50 nM Tris pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, and 0.02 mg/ml each of aprotinin, leupepsin, and pepstatin). Lysates were sonicated and clarified by centrifugation. Protein was quantitated by Bradford analysis and 20–100 μg protein per lane resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Transfer and blotting was as described (Dulic *et al.*, 1992). Proteins were detected by electrochemiluminescence (ECL). Densitometry was performed using the Molecular Dynamics Imaging system and ImageQuant software to quantitate the relative amounts of p27 protein detected on Western blots. For detection of cyclin E-associated proteins by immunoprecipitation-Western analysis (IP-Western), cyclin E was immunoprecipitated from 200 μg protein lysate, complexes resolved, blotted and blots reacted with cyclin E, cdk2, p21, and p27 antibodies. For detection of cdk4 complexes, 100–400 μg protein lysate was immunoprecipitated with cdk4 antibody and associated cyclin D1, p15, p16, p21, and p27 proteins detected by immunoblotting. For immunodepletion of p27, p27 was serially immunoprecipitated three times from 200 μg protein lysate and then cyclin E was immunoprecipitated from the p27-depleted lysate. The amounts of immunoprecipitable cyclin E protein, associated cdk2 and p27 proteins, and kinase activities prior to and after p27 immunodepletion were compared using IP-Western blotting and IP-kinase assays.

Oligonucleotide transfections

The sequences of the GS5422 antisense (ASp27) and mismatch p27 GS5585 (MSp27) C-5-propyne modified phosphorothioates used were 5′-TGGCTCTCXTGCGCC-3′ and 5′-TGGCTCXCTTGCGCC-3′, respectively. X indicates the proprietary 'G-clamp' modification of these oligonucleotides, provided by Gilead Sciences. Asynchronously growing cell cultures were transfected with oligonucleotides at a concentration of 50 nM using cytofectin G3815 at 2.5 μg/ml, as described (St.Croix *et al.*, 1996). After 6 h the transfection cocktail was removed and replaced with complete medium containing 100 nM DHT. Cells were recovered for flow cytometric and protein analysis 36 h thereafter. 100 nM DHT-arrested cells were also transfected with ASp27, however, the reduction of p27 was considerably less in these cells than after transfection of ASp27 into asynchronously growing cells. The minor reduction in p27 protein levels in these DHT arrested cells was not sufficient to cause release from G0.

Cyclin-dependent kinase assays

Cdk4 kinase assays were performed using the method of Matsushime *et al.* (1994), using a truncated recombinant retinoblastoma protein as substrate. Quantitation of radioactivity incorporated in the substrate was performed using a Molecular Dynamics PhosphorImager and ImageQuant software. Cyclin E-associated kinase assays were performed using either a monoclonal anti-cyclin E antibody (mAb E172, from E Harlow, Mass. General, MA, USA) or a polyclonal anti-cyclin E antibody (from D Agarwaal, Lee Moffat Cancer Center, FL, USA). Histone H1 was used as substrate for cyclin E-associated kinase assays. In each case background activity was determined using a non-specific antibody as a control. Background activity was subtracted, and kinase activities were graphed as a per cent maximum activity.

Antibodies

The following antibodies were used in the immunoblotting experiments: pRB mouse mAb from Pharmingen; cdk2 rabbit pAb sc-163, cdk4 rabbit pAb sc-260, cdk6 rabbit pAb sc-172, cyclin D1 mouse mAb HD11, cyclin A rabbit pAb sc-596, cyclin B1 mouse mAb sc-245, and p21 rabbit pAb sc-397, all from Santa Cruz Biotechnology, CA, USA; p27 mouse monoclonal antibody from Transduction Laboratories, Lexington, KY, USA. PSA antibody was purchased from DAKO, Denmark. Monoclonal PSTAIRE antibody was a gift from S Reed (The Scripps Research Institute, CA, USA); cyclin D1 mouse mAb DCS-11 and p16 mouse mAb DCS-50, from J Bartek (Danish Cancer Society, Denmark); E12 and E172, mouse monoclonal antibodies to cyclin E from E Harlow (Mass. General, MA, USA); and cdk4 rabbit polyclonal antibody, for use in immunoprecipitation of cdk4, was provided by D Beach (CSH Labs, NY, USA). A monoclonal antibody, JC-6, which recognizes the third ankyrin repeat of human p16 (Enders *et al.*, 1995) and cross reacts with human p15, was used for immunoblotting of p15 in these studies. Results were confirmed by repeat biologic assays with different cell lysates.

Acknowledgments
We thank Jeff Donovan for helpful discussions and advice in the design of the antisense p27 experiments. This work was funded by a grant from the US Army Department of Defense Prostate Cancer Research Program to JM Slingerland. J Tsihlias was supported by a Terry Fox Fellowship from the National Cancer Institute of Canada. JM Slingerland is supported by Cancer Care Ontario.

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APPENDIX 2
FIGURES 1-9

Figure 1

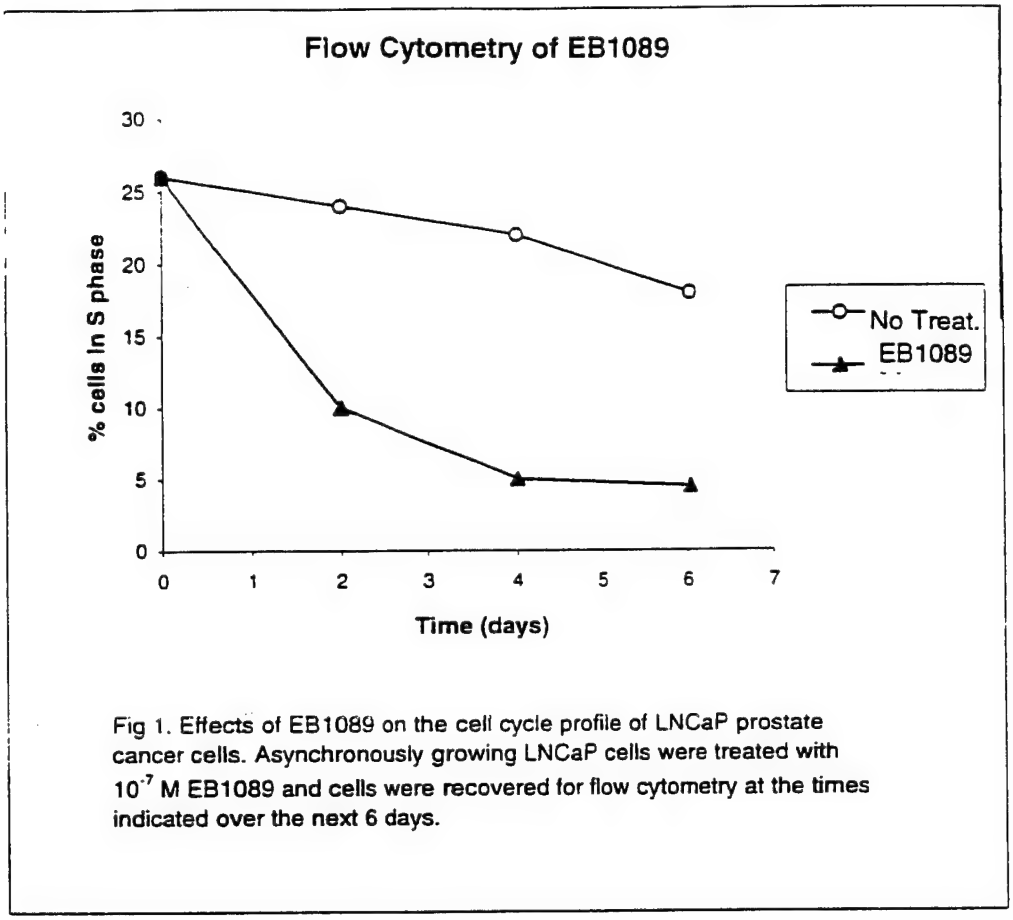

Fig 1. Effects of EB1089 on the cell cycle profile of LNCaP prostate cancer cells. Asynchronously growing LNCaP cells were treated with 10^{-7} M EB1089 and cells were recovered for flow cytometry at the times indicated over the next 6 days.

EB1089 effects on cell cycle regulators

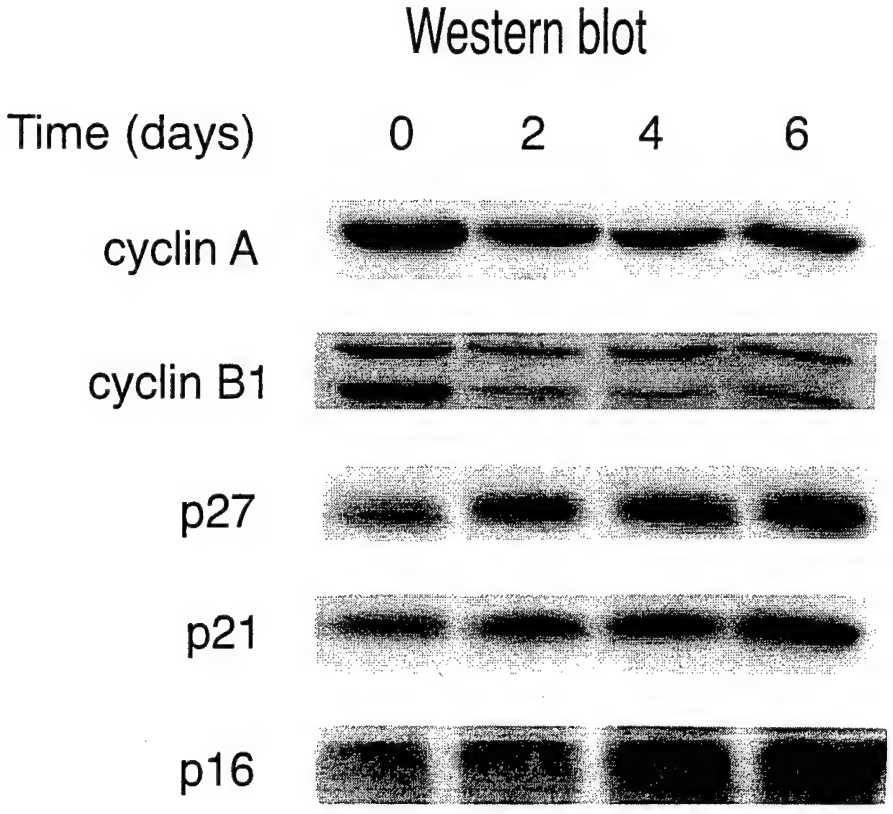

Fig 2. Effect of EB1089 on cell cycle regulators. As cells entered G1 arrest over 6 days of drug treatment, levels of cyclin A and cyclin B1 fell and the cdk inhibitors p21, p27 and p16 increased. Levels of cyclin D1, cyclin D2, cyclin E and cdk2, cdk4 and cdk6 did not change (not shown).

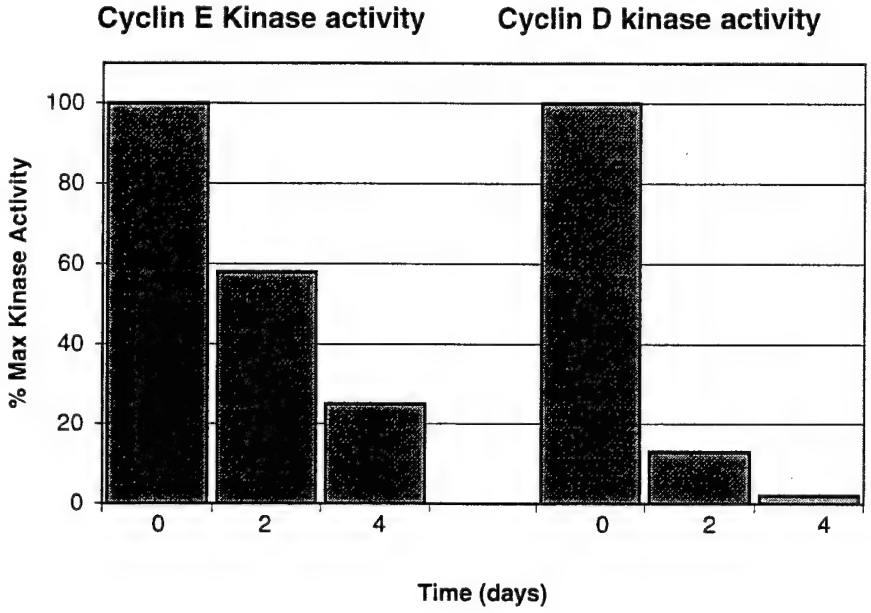

Fig 3. Effects of EB1089 on G1 cyclin-cdk activities. Over 4 days of treatment with EB1089 10^{-7} M, cyclin E- and cyclin D1-dependent kinase activities were inhibited.

EB1089 10-7 M

IP p21

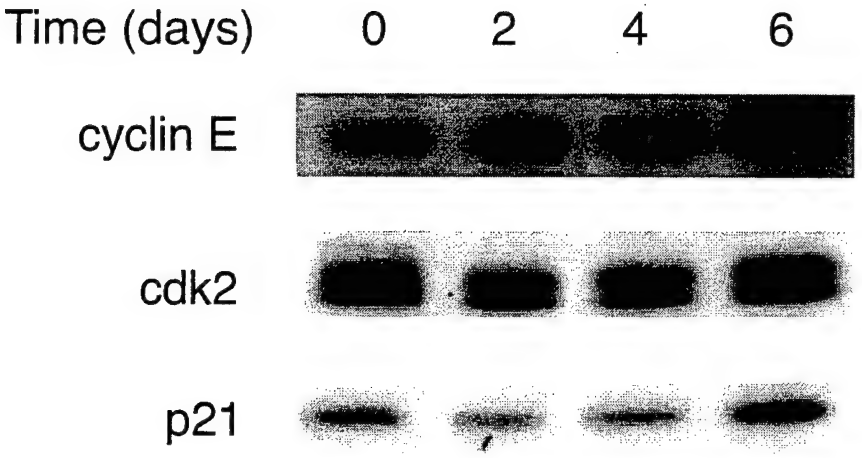

Fig 4. EB1089 causes an increase in p21 and an increase in p21 binding to cyclin E-ckd2. p21 was immunoprecipitated from cell lysates before (T=0) and 6 days after EB1089 treatment. Complexes were resolved on SDS-PAGE, blotted and p21-associated proteins detected by immunoblotting.

EB1089 10-7 M

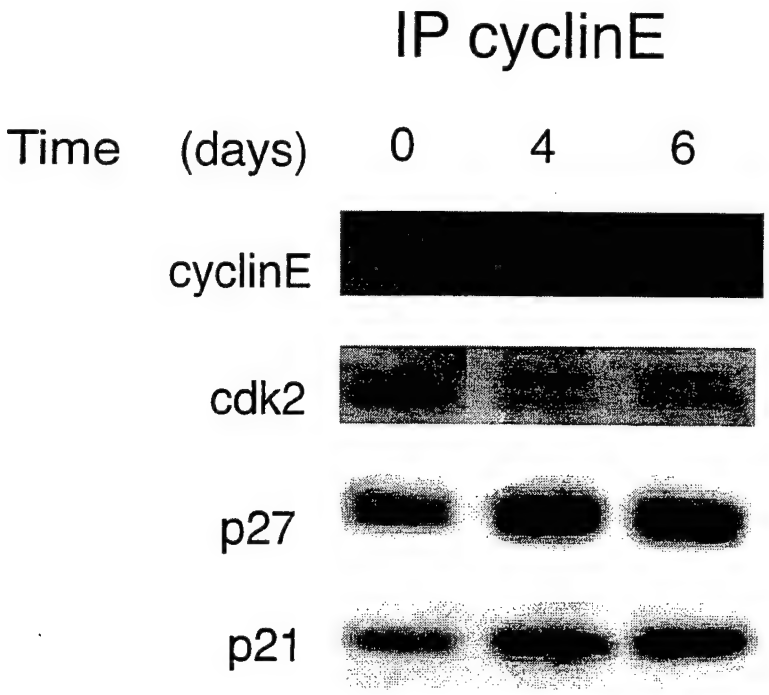

Fig 5. Binding of cdk inhibitors p21 and p27 to cyclin E-cdk2 is increased by EB1089. Over 6 days of EB1089 treatment, levels of cyclin E-bound p21 and p27 increased significantly. Cyclin E levels were not changed by EB 1089 treatment.

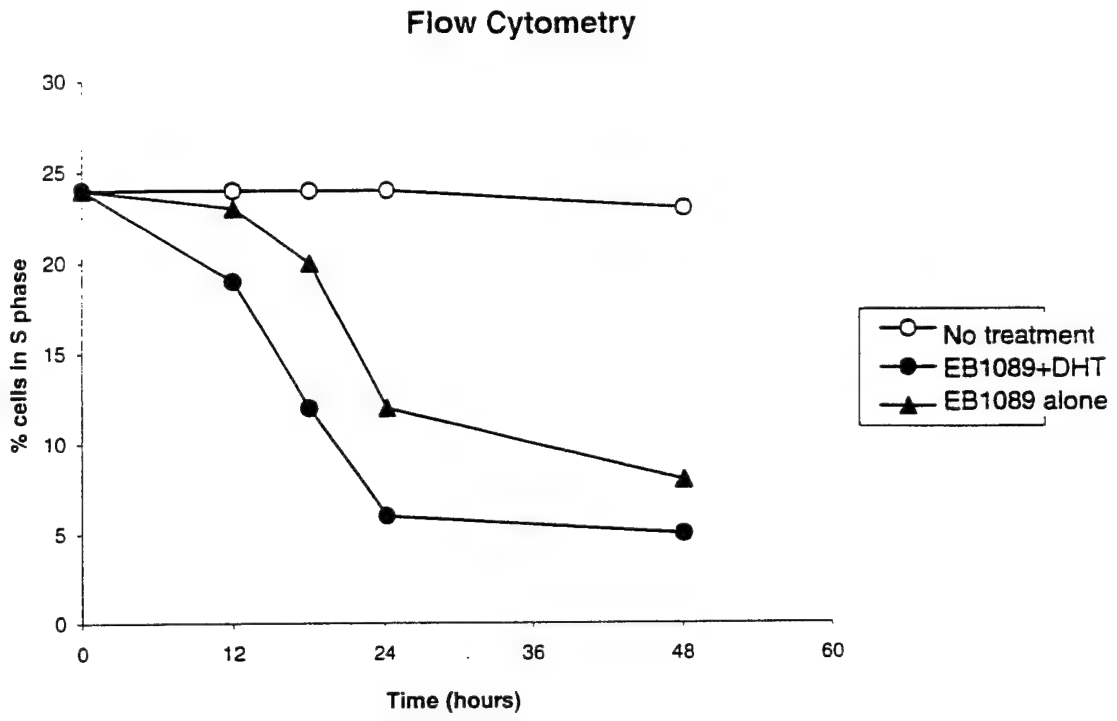

Fig 6. Asynchronous cultures of LNCaP were treated with EB1089 10^{-7} M alone, EB1089 10^{-7} M+DHT 0.3 nM, DHT 0.3 nM alone (not shown), or no drug. Cells were recovered at the indicated times for flow cytometric analysis. The % cells in S phase are graphed with time. DHT alone caused essentially no change in the cell cycle. EB1089 with DHT caused more rapid and profound inhibition of cell cycle progression than did EB1089 alone.

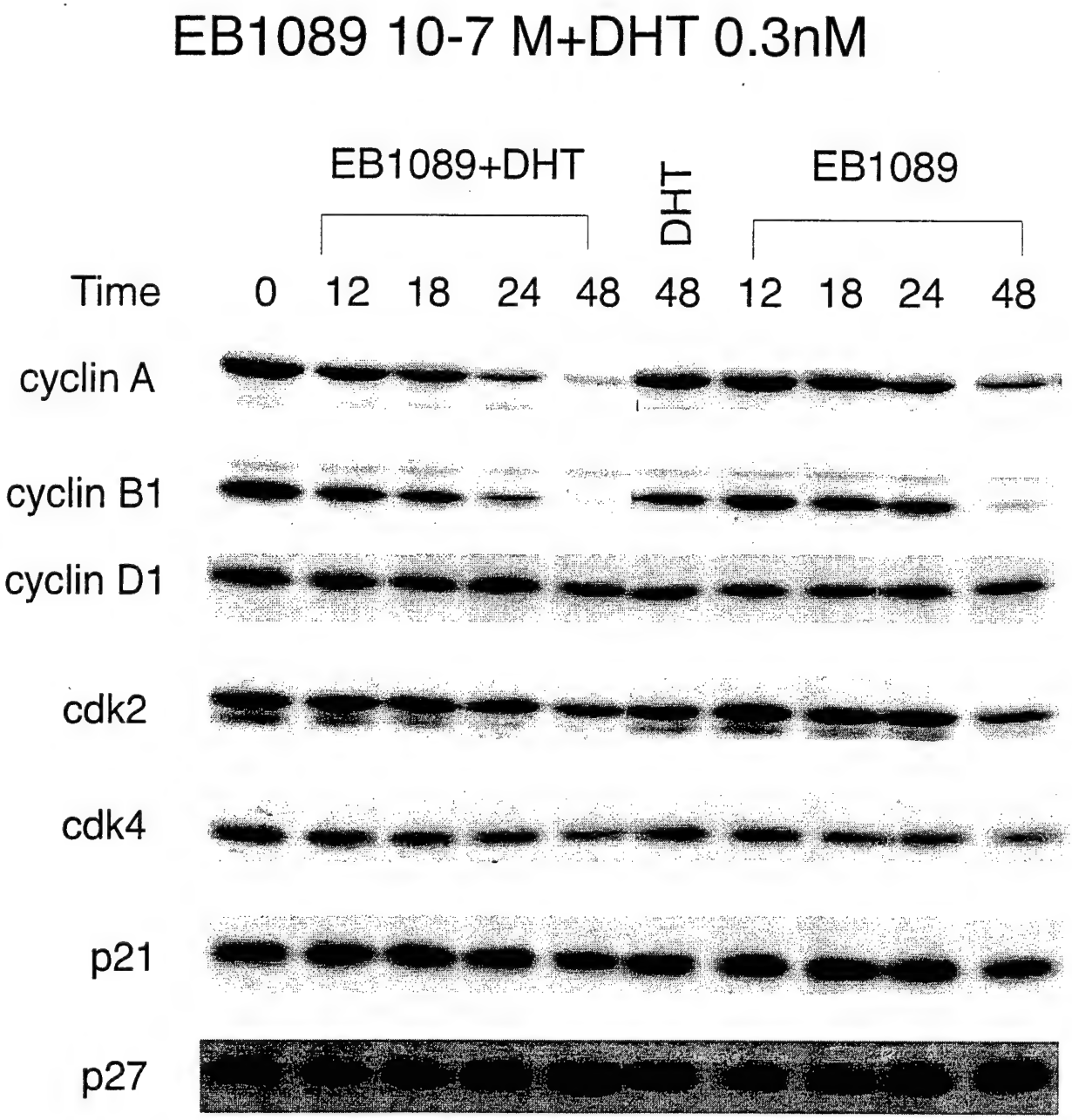

Fig 7. Comparison of effects of EB1089 alone and EB1089 with DHT on cell cycle regulators. The combination of EB1089 10-7 M with 0.3 nM DHT caused a more rapid loss of cyclins A and B1, and a more rapid rise of p27 than did EB1089 alone. Levels of cdk2, cdk4, cyclin D1 and cyclin E were not affected significantly by either drug treatment. There was little change in p21 within 48 hours. While p21 levels rose with EB1089, the increase was most notable at 4 to 6 days (not shown).

EB1089 10-7 M+DHT 0.3nM

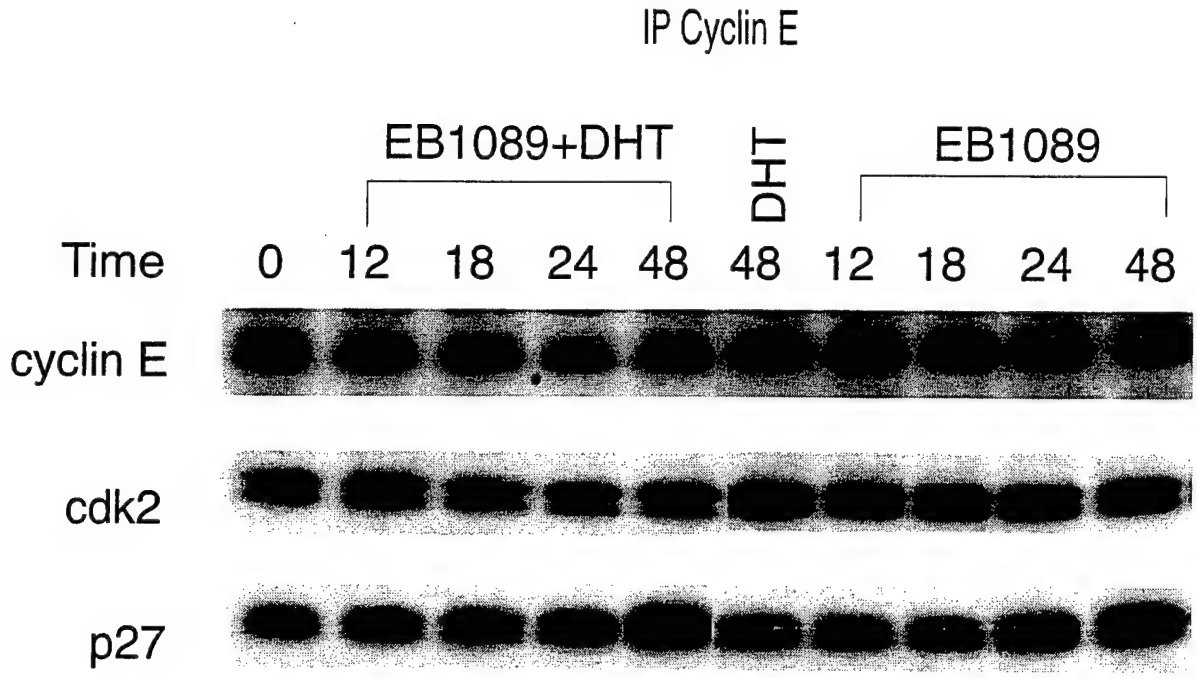

Fig 8. EB1089 and DHT cause a greater increase in p27 binding to cyclin E-cdk2 than EB1089 alone. At intervals following addition of the indicated drugs, cells were lysed and cyclin E immunoprecipitated. Cyclin E immune complexes were resolved and associated proteins were detected with the indicated antibodies. There was a 5 fold increase in p27 binding to cyclin E in the EB1089+DHT treated cells. p21 did not increase in cyclin E complexes during the first 48 hours in any treatment group.

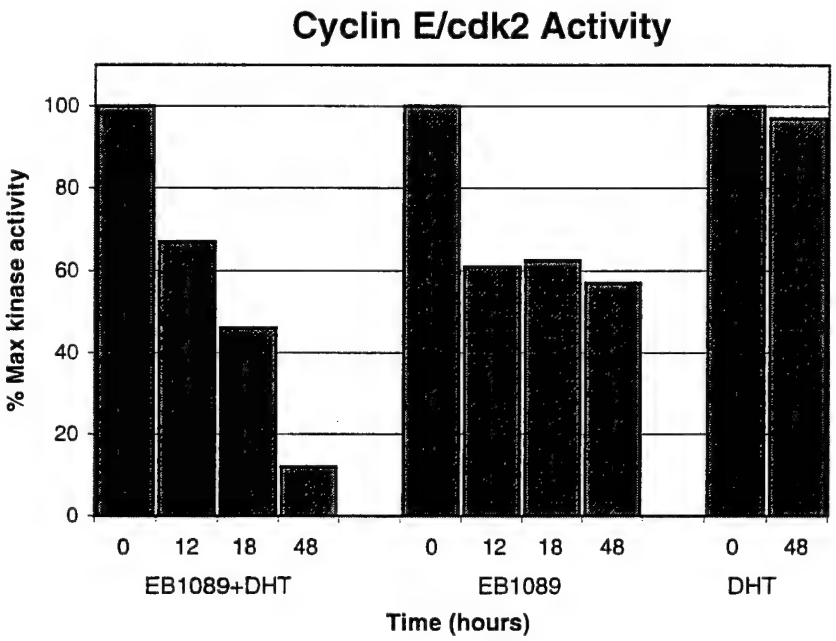

Fig 9. Drug effects on cyclin E-cdk2 activities. The inhibition of cyclin E-cdk2 by EB1089+DHT was more rapid and more profound than that caused by EB1089 alone. There was essentially no inhibition of cyclin E-cdk2 activity by DHT 0.3 M alone.

APPENDIX 3

SUNNYBROOK HEALTH SCIENCE CENTRE

Animal Use Protocol Modification Application

For Office Use Only

Expiry Date: 14 Jun 00

Protocol#: 99-029

Complete sections 1 & 2 in FULL. For all other sections, document only the information which has CHANGED from the approved protocol.

1 Principal Investigator

Surname	First Name	Initial
Slingerland	Joyce	M

Department/Faculty: Cancer Biology Research

Business Telephone	Laboratory Telephone	Residence Telephone
(416) 480-6100 Ext.3494	(416) 480-6100 Ext.3495	(416) 924 2725

Pager	E-mail Address
(416) 232 7077	joice.slingerland@utoronto.ca

2 Project

Project Title

In vivo assays of growth suppression of porstate cancer xenografts by vitamin D3 analogs and dihydrot estosterone.

3 Funding Changes

Internal/External	Peer Reviewed?	Name of Agency/Source

4 Emergency Contact Changes

	Rank	Emergency Telephone
A.Faculty - Name (mandatory)		
B.Other (optional)		

5 Research Staff Changes

Name	Add/Remove	Position	Training
			☐
			☐

6 Experimental Design Changes

Describe modification to experimental design: Is this a change to an existing procedure? ○ or an addition? ☒

Terminal blood collection will be done in some mice by cardio centesis using a 1 ml syringe and a #23 gauge needle under anaesthetic.

Outline reason(s) for the modification

We require a minimum sample size of 0.5 ml in order to have the blood tested.

7 Change in Animals

Animal Strain	# of Animals	Sex	Age/Weight	Source of Animals	Location of Holding	Location of Experiment

8 Signatures

Principal Investigator or Course Director — Signature: Joyce — Date: Oct 28/99

Chairperson Local Animal Care Committee — Signature: [illegible] — Date: Jan 11/00

Veterinarian — Signature: [illegible] — Date: / /

Animal Use Protocol

IMPORTANT! Incomplete protocols will be returned!

For Office Use Only

New Protocol# 99-029

Expiry Date 14 Jun 00

(●) New Project ( ) 4th yr. Renewal Previous Protocol#

1 Principal Investigator or Course Director

Surname	First Name	Initial	Personnel No.
Slingerland	**Joyce**	**M**	

Department/Faculty: **Cancer Biology Research**

Position/Rank: **Senior Scientist**

Business Telephone	Laboratory Telephone	Residence Telephone
(416) 480-6100 Ext. 3494	**(416) 480-6100 Ext. 3495**	**(416) 924 2725**

Pager: **(416) 232 7077**

E-mail Address: **joyce.slingerland@utoronto.ca**

Mailing Address: **Sunnybrook & Women's College Health Sciences Centre, S218, 2075 Bayview Ave., Toronto, ON M4N 3M5, Canada**

NAME OF DESIGNATED ALTERNATE FOR EMERGENCIES

A.Faculty - Name (mandatory)	Rank	Emergency Telephone
Jorge Filmus	**Senior Scientist**	**(416) 406 0627**
B.Other (optional)	Rank	Emergency Telephone

2 Project

Project Title

In vivo assays of growth suppression of prostate cancer xenografts by vitamin D3 analogs and dihydrotestosterone.

3 Funding

(●) External Agency ( ) Internal Source

Grant Account # 724179322

Status: (●) Awarded ( ) Pending ( ) Peer Reviewed (by whom?)

(●) Peer Reviewed ( ) Non-Peer Reviewed ( ) Non-Peer Reviewed

4

	Day	Month	Year		Day	Month	Year
PROPOSED START DATE	15	June	1999	EXPECTED COMPLETION DATE	15	June	2000

5

CATEGORY OF INVASIVENESS ( ) A ( ) B ( ) C (●) D ( ) E

6 Declaration

TYPE OF EXPERIMENT (Check all applicable boxes) [X] Research [ ] Teaching [ ] Testing [ ] Research/Teaching

SURGICAL [ ] Acute [X] Survival NON-SURGICAL [ ] Acute [ ] Chronic

All animals in this protocol will be maintained and used in accordance with the current recommendations of the Canadian Council on Animal Care, the requirements under the Animals for Research Act, R.S.O 1980, and Sunnybrook Health Science Centre Animal Care Committee Policies and Guidelines

Approvals

Principal Investigator or Course Director: **Joyce M. Slingerland** Signature: J. Slingerland Date: **June 3, 1999**

Chairperson Local Animal Care Committee Signature: M. Johnston Date: Sept 29/99

Veterinarian Signature: [illegible] Date: 29/09/99

In LAYMAN'S TERMS, BRIEFLY describe all procedures and techniques to be used; giving a DETAILED description of ONLY those performed on animals. Space for this description is limited; attach 1 ADDITIONAL PAGE if necessary.

Lay Description

I. RATIONALE

One of the major forms of therapy for advanced, metastatic prostate cancer is androgen ablation. This therapy aims to reduce the available amount of male hormone or androgen that can stimulates growth of prostate cancer cells. This is accomplished either by surgical removal of the testes (orchidectomy) or by a medical orchidectomy using various forms of medication that block the effects of androgen. Unfortunately, blockade of androgen effects in men has significant negative side effects.

Over the last year and a half we have been studying the growth inhibitory effects of high dose androgen or dihydrotestosterone, and of vitamin D3 analogs including an analog EB1089, produced by the Leo Co. in Denmark. Both of these agents, used on their own, cause growth arrest in tissue culture of the prostate cancer cell line LNCaP. However, the dose of androgen or DHT that causes growth arrest in of prostate cancer in tissue culture is much higher than the normal level in the human male. On their own, low doses of dihydrotestosterone do not inhibit prostate cancer growth. Recently, we have found that when a low, physiologic dose of dihydrotestosterone is added to the usual dose of vitamin D3 analog EB1089, the two drugs act synergistically to cause amore rapid and more complete arrest of prostate cancer cells growth. We wish now to explore the possibility that a combination of low, physiologic doses of dihydrotestosterone with EB1089 may mediate arrest of prostate cancer cells in an experimental model of human prostate cancer, that is human prostate cancer cells grown in an immunodeficient or nude mice . We plan to ascertain whether this low, physiologic dose of dihydrotestosterone, administered through an indwelling subcutaneous pellet to the mouse and oral administration of EB1089 can either prevent the development of prostate cancer tumors when these are injected subcutaneously in the mouse, or can inhibit the growth of tumors once they are established. Studies of this nature in the mouse are essential to provide the data that would allow the next step, which is to proceed with clinical trials of these novel forms of relatively innocuous hormones in human prostate cancer patients. This vitamin D3 analogs have been specifically designed to inhibit prostate cancer growth and yet they do not cause the negative side effects of hypercalcemia, that is, the increase in serum calcium that is normally caused by vitamin D3 itself. Low dose dihydrotestosterone is currently in use clinically to prevent osteoporosis, the loss of muscle mass and the loss of libido that accompany human male aging. Low dose androgen is currently not used for prostate cancer except in certain clinical trials, because of its potential to promote prostate cancer growth. We wish to explore the possibility that, in combination with vitamin D3 analogs such as EB1089, low dose androgen may have its positive side effects on the human subject and rather than promoting prostate cancer growth, may indeed have a synergistic effect to inhibit prostate cancer growth. We have documented extensively in tissue culture this growth inhibitory effect on prostate cancer over the last year and a half. Currently EB1089 is being used in clinical trials for prostate cancer in humans. The LNCaP and Shionogi cancer cell lines have been chosen for use in these experiments because they are sensitive to androgen and vitamin D3, and also because they form subcutaneous nodules when injected into the mice strains indicated below. They do not metastasize. Although these tumors will grow as subcutaneous nodules, the fact that they do not have a tendency to spread to other organs in the mouse will minimize animal suffering. The subcutaneous tumors that arise do not cause the animals to be systemically sick.

II METHODS

A Cell Lines. Human prostate cancer LNCaP cells, passage 40, are grown in RPMI 1640 with 5% fetal bovine serum (FBS). The Toronto subline of the transplantable SC-115 Shionogi mouse mammary carcinoma will be used.

B In Vivo Tumour Growth. 6- to 8- week old male athymic nude mice (Crl:nu/nu(CD-1) BR) will be inoculated sc with 1 x 10^6 LNCaP cells suspended in 100µL Matrigel. 2 x 10^7 Shionogi cells will be injected into male DD/S mice. Tumours are measured twice weekly and their volumes calculated by the formula L x W x H x 0.5236 (7). Blood samples for sequential DHT and PSA measurements will be obtained as previously described. Blood will be taken from the tail vein of the mice for serum calcium and for PSA and testosterone levels. Samples will not exceed 0.2 ml and there will be a waiting time of two weeks between sample takings.

C Surgical Castration. Inject 0.20-0.25 ml of anesthesia IP. Wipe belly and lower abdomen with iodine swab. When mouse does not respond to pinching or squeezing his foot, make incision midline through skin. Cut through muscle wall being careful not to cut colon. Using forceps, pull left testicle and epididymal fat pad

out of body. Tie three knots below testicle and fat pad with 3.0 chromic gut thread. Cut off testicle and fat pad above knots. Repeat for right testicle and epidydimal fat pad. Stitch muscle wall by pulling a needle and 3.0 chromic gut thread through muscle on both sides of the incision and making three knots. Repeat at another point along the incision. Be careful not to pierce internal organs. Clamp skin together with 1 or 2 staples. The staples should be removed after 10-14 days. The post-doctoral fellow who will do these experiments is a urologic surgeon.

D Histology and Immunohistochemistry. Tumors will be excised from sacrificed mice and processed for histological analysis as previously described . Deparaffinized and frozen sections will be incubated with monoclonal antibodies prepared against PSA, p27, and MIB1 (Ki-67), and digoxigenin-linked nucleotide and N-terminal deoxynucleotidyl transferase (Apotag, Oncor, Inc.) Frozen section will also be used for in situ hybridization to analyze changes in mRNA levels and distribution of these markers when appropriate.

E Western Analysis. Tumour specimens harvested at various times pre- and post-castration and following treatment with DHT and EB1089 will processed for cytoplasmic and nuclear protein extraction and probed for antibodies against AR,p27, VDR, and actin as a loading control.

F RNA Isolation and Northern Blot Analysis. Gene expression will be tested by Northern analyses using standard techniques.

G Cell cycle profiles. The distribution of tumor cells in the different cell cycle phases will be monitored using tumor dissociation according to the Hedley method and routine preparation for Facs analysis.

II. EXPERIMENTAL DESIGN.

A. Determination of physiologic DHT doses and normocalcemic doses of EB1089 in mice. Several mice will be used to assay the serum levels of DHT following implantation of subcutaneous DHT pellets in castrate mice. We will use 12.5 mg subcutaneous pellets which should provide serum levels of 3-5 ng/ml in the mouse. Our goal is to approximately the serum levels of DHT in non-castrate mice without DHT pellets (eg 3-5 ng /ml serum). While EB1089 does not cause hypercalcemia in humans, it will be necessary to demonstrate that the doses recommended by the manufacturer, Leo, for use in mice (0.1-1.0 μg/kg/day) do not cause any negative side effects, including hypercalcemia in the mice. Both assays of DHT and calcium can be performed in the hospital's biochemistry department.

B. Effects of DHT and EB1089 to suppress tumor formation

We will test whether low dose DHT(delivered by sub-cutaneous pellet) and EB1089 can prevent tumor formation as follows. Mice will be injected at a maximum of 4 sites (max. vol. of 200 μl/site) sc with the respective tumor lines after a 2 week pre-treatment with DHT, DHT +EB1089, EB1089 alone or no treatment (10 mice each treatment category). Hormonal treatments will continue for the duration of the experiment. Tumor number and size and markers of proliferation and DNA analsysis will be monitored at the termination of the experiment. The termination of the experiment will be defined as follows: Animals will be sacrificed 6 months after tumors arise or when the tumor reaches a mean subcutaneous flank tumor diameter of 17 mm, that is a volume exceeding $(17\ mm)^3$. Any animal bearing an ulcerated tumor will be sacrificed as soon as ulceration is detected.

C. Effects of DHT and EB1089 to suppress established tumor growth

DHT supplemented castrate mice will be injected at 4 sc sites per animal as above with either LNCaP or Shionogi cells and tumors allowed to establish over a 4 wk. Period. We anticipate tumor sizes of <0.5 cm after 4 wks. After 4 weeks , hormonal treatment will begin. 10 Animals will be placed in each of 4 experimental arms: 1) no treatment controls 2) DHT 3) EB1089 4) both DHT and EB1089. Tumor size and parameters of proliferation and p27 levels will be monitored at 1, 2, 3, and 4 months or until the tumor reaches a mean subcutaneous flank tumor diameter of 17 mm whichever occurs first. Two mice will be sacrificed and tumors will be harvested prior to and 1, 2, 3 and 4 months after their appearance and divided into 3 portions: an unfixed portion with be used for Facs analysis; one portion will be fixed in 10% NBF and embedded in paraffin for morphological analysis; a third portion will be frozen at -80°C for Western blots and for RNA extraction and northern analyses. H&E and Apotag staining will be used to evaluate castration-induced cell death with characteristic apoptotic cells. Immunostaining using antibodies directed against the markers mentioned above will be performed to evaluate qualitative and semi-quantitative (distribution and intensity) changes in respective protein levels following hormonal therapies (DHT and or EB1089). Internal reference slides will be used to normalize for variation in intensity of immunostaining between preps.

8 Objectives

PRIMARY OBJECTIVE(S) OF THIS PROJECT
Summarize the primary objective(s) and benefits expected from the study in terms suitable for NON-scientific readers.

We have found that a combination of low, physiologic doses of dihydroxytestosterone and vitamin D3 analogs cause a synergistic growth inhibitory response in prostate cancer cells in culture. We wish to test whether low dose dihydrotestosterone and vitamine D3 analogs given concurrently to mice can prevent the growth/development of prostate cancer xenografts or can cause xenograft tumors, once established, to regress.

9 Research Staff

NOTE: University of Toronto 1-day course is MANDATORY for new graduate students, research technicians and technologists, research assistants/associates, and post-doctoral fellows.
*A box with an "x" indicates that the staff member has completed or is registered for the U of T Animal Care Course, or equivalent training.

Name	Department	Position	Training*
William Zhang	Cancer Biology-Research	Post-Doctoral	☒
Joyce Slingerland	Cancer Biology Research	Senior Scientist	☒
			☐
			☐
			☐
			☐
			☐

10 Animals to be Used

Animal Species (Common Name)	Strain	# of Animals	Sex	Age/Weight	Source of Animals	Location of Holding	Location of Experiment
Mice	CB17SCID	130	M	4-8 wks	Charles Rivers	SB	SB
	DD/S	130	M	6-8 wks	Charles Rivers	SB	SB

ANIMAL HOUSING REQUIREMENTS ○ Standard Cages ◉ Microisolator Cages ○ Other

11 Justification

JUSTIFICATION FOR: A. Species and B. Number of Animals Used Please limit justification to the space provided.

A. CB17 SCID mice will be used for xenografting the human prostate cancer line LNCaP. Syngeneic DD/S strain mice will be used for the in vivo tumor studies with the murine Shionogi hormone sensitive cancer line.

B. We propose to use 10 animals from each strain (CD17 SCID) and DD/S) to assay baseline effects of castration and use of DHT s.c. pellets on DHT levels. Vitamin D3 analogs may cause hypercalcemia. We will ensure that doses of EB1089 administered po (0.1-1.0 mg/day) do not cause hypercalcemia in the experimental animals. Physiologically equivalent doses do not cause hypercalcemia in humans. The 2 experiments detailed in Part 7 A involves 10 animals in each of the 4 arms for each of the LNCaP and Shionogi cell line experiments. Part 7B involves 20 mice in each of the 4 arms for each of the 2 cancer cell lines. We estimate that this number is probably the minimum required to allow meaningful interpretation of results.

12 ALTERNATIVES Are non-animal alternatives available for this project? ◯ Yes ⊗ No

If Yes, explain why they have been rejected.

Alternatives

This project is specifically designed to test, in an animal model, growth inhibitory effects of EB1089 and DHT on prostate cancer that we have studied extensively already in tissue culture. The next step in moving these relatively innocuous hormonal treatments into clinical trials for human prostate cancer, is to test these drugs on prostate cancers in an animal model.

13 Drugs Used

DRUGS USED FOR ANAESTHESIA/ANALGESIA	DRUG	DOSAGE	ROUTE OF ADMINISTRATION
A. Pre-Anaesthesia			
Pre-Anaesthesia			IP
B. Anaesthetic Induction:	ketamine , xylazine		IP
Anaesthetic Maintenance:			
C. Analgesic(s)			
D. Other			
Other			
E. None ☐			

14 Euthanasia

⊗ Anaesthetic Overdose (specify agent) _____ ◯ Carbon Dioxide

☒ Cervical Dislocation (small rodents only) ◯ Decapitation

◯ Exsanguination (under anaesthesia) _____

◯ Other (specify) _____

15 Hazardous Agents

☐ Biological (specify) _____ Biohazard Safety Committee Approval

☐ Chemical Occupational Health & Safety Dept. Approval

☐ Carcinogen Occupational Health & Safety Dept. Approval

☐ Radioisotope/Radiation R/A Permit # Expiry Date

☒ None

ANIMAL CARE COMMITTEE USE ONLY - ADDITIONAL NOTES

Note: JetForm 4.x users must manually open the file, "tis.mdf", in the "aup" directory/folder to use the Transgenic Info. Sheet.

Animal Use Data Form

Completion of this form is a requirement of the Canadian Council on Animal Care (CCAC).

For a protocol to be considered by the SHSC Animal Care Committee, this form must be completed and submitted with the protocol form.

Institution Name: Sunnybrook Health Science Centre **Institution Code:** MH02

Protocol#	Category [1]	Protocol Description [2]	PAU [3]	Species [4]	AR [5]	AU/Yr
99-029	D	An investigation of whether prostate cancer tumors grown subcutaneously in castrated mice can be prevented or growth inhibited by vitamin D3 analogs and dihydrotestosterone.	2	Mice	260	For Office Use Only

1. Category:	Category of Invasiveness
2. Protocol Description:	Please give a descriptive protocol title that indicates, in LAY TERMS, the nature of the procedures used (preferably in 40 words or less)
3. PAU	Purpose of Animal Use: 1. Studies on a fundamental nature in sciences relating to essential structure or function 2. Studies for medical purposes, including veterinary medicine, that relate to human or animal disease or disorders 3. Studies for regulatory testing of products, for the protection of humans, animals, or the environment 4. Studies for the development of products or appliances for human or veterinary medicine 5. Education and training of individuals in post-secondary institutions or facilities
4. AR	Number of Animals Requested
5. AU/Yr	Number of Animals Used / Year To be completed at the end of each calendar year (If a group of animals is reused in a second protocol, please indicate an "R" and the 1st protocol number next to the number of animals used for the 2nd protocol).